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THE EFFECTS OF 2560 r OF X-RAYS ON SPERMATOGENESIS IN THE MOUSE[1]

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The effects of irradiation on the mammalian testis have been the subject of numerous investigations (for example Regaud and Blanc, 1906; Hertwig, 1938; Eschenbrenner and Miller, 1950; Oakberg, 1955; and Bryan and Gowen, 1956). The more recent papers of this series have been concerned with quantitative aspects of the problem. The foregoing studies have established the fact that the spermatogonia are the most radiation-sensitive constituents of the seminiferous tubules. After exposure to radiation, spermatogonial proliferation is progressively reduced and the frequency of spermatogonia declines to a very low level. Following this irradiation-induced decrease in spermatogonia, the other cell types (spermatocytes, spermatids and sperm) disappear in the order of their development. Knowledge of the nature of the spermatogonial response is therefore of considerable importance to an approach toward an understanding of the action of irradiation on cells and tissues. Evidence has accrued which suggests that spermatogonial necrosis may be an important factor (Regaud and Lacassagne, 1927; Hertwig, 1938; and Oakberg, 1955). In this regard conclusions based on tracer studies must also be considered. The studies of Holmes (1947), Howard and Pelc (1953), Forssberg and Klein (1954), Smellie *et al.* (1955) and others, clearly show that irradiation effectively inhibits DNA synthesis together with mitotic activity. Furthermore the data of Howard and Pelc (1953) indicate that the mitotic inhibition (or delay) is brought about by the failure of cells in interphase to enter the synthetic phase, rather than by interruption of synthetic processes already going on. These studies would suggest that in the case of the testis, the cessation of spermatogonial activity (through inhibition of DNA synthesis) should be a major factor in bringing about the irradiation-induced depletion of spermatogonia. The data of Bryan and Gowen (1956), derived from quantitative histological and from cytophotometric studies, are in accord with these ideas—as are the earlier conclusions of Eschenbrenner and Miller (1950) and Shaver (1953). There are, then, two rather different responses to irradiation which have been advanced as explanations for the observed behavior of irradiated mammalian seminiferous tubules. The important

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point is: what are the relative levels of importance which may be ascribed to either process? It appeared likely that a comparison of results following exposure to different dose levels of x-rays would shed more light on the nature of any relationship between these proposed mechanisms. In our previous paper results obtained following exposure to 320 r of x-rays were reported. This present paper reports data obtained following exposure to a high dose of x-rays (2560 r). These data are, where feasible, presented together with corresponding data from our 320 r experiment. As will be seen, the available evidence suggests that both mechanisms play a role in the observed radiation response of spermatogonia, the level of importance ascribable to either one depending upon the dosage levels of radiation employed.

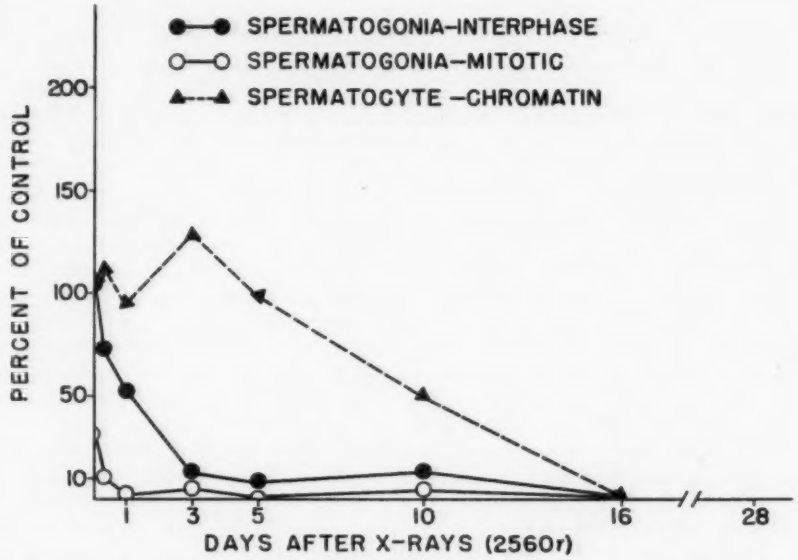

FIGURE 1. Strain Ba. Incidence of spermatogonia and spermatocytes at different times following 2560 r of x-rays.

MATERIALS AND METHODS

The animals chosen were 58-day-old males of strains BALB/Gw (hereinafter referred to as Ba) and S. These inbred strains of mice differ in their sensitivity to mouse typhoid. The experimental animals were irradiated in plastic tubes and were exposed to a dose of 2560 r (250 pkv, 30 ma; filtration 0.25 mm. Cu, 1 mm. Al; anode-target distance 47.5 cm., dose rate 430 r/min.). The irradiation was delivered to the pelvic region only, the rest of the body being shielded with lead. These conditions of irradiation are, except for the x-ray dose, identical with those of our previous studies (Bryan and Gowen, 1956).

Control and irradiated animals were killed at 1, 8 and 24 hours, 3, 5, 10, 16

and 28 days following exposure. The 28-day material is missing from the S series due to death of animals prior to this sampling time. From each animal the testes were rapidly removed and weighed. One testis was then fixed in Carnoy's acetic-alcohol (1:3) and the other used for dry weight determinations.

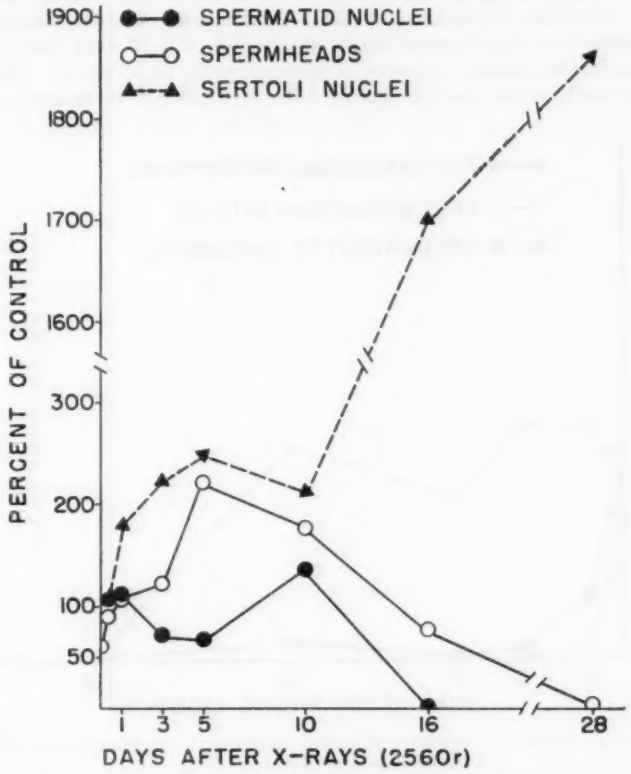

FIGURE 2. Strain Ba. Incidence of spermatids, sperm and Sertoli nuclei at different times following 2560 r of x-rays.

The histological material was processed, and slides were stained, as described earlier (Bryan and Gowen, 1956). As in our previous work, the procedure of Chalkley (1943) was used to obtain estimates of the relative areas of the tubules occupied by each stage of spermatogenesis. This procedure was also used to provide data with respect to spermatogonial and non-spermatogonial necrosis during the first 24 hours following exposure to x-rays.

RESULTS

The data obtained are summarized in Tables I–III. Data pertaining to both strains are presented together for ease of comparison. In Table I all values are

expressed in terms of per cent of control values. These data are also expressed in graphical form in Figures 1–4.

The data of Table I indicate that the relative area occupied by spermatogonia in interphase undergoes little change during the first hour after irradiation. Thereafter there is a pronounced and progressive decline which reaches a low point by 5 days following exposure. The data further suggest that there may be an abortive attempt at regeneration during the period of 5–10 days after x-rays, followed by a further decline (absence of spermatogonia at 16 days). With respect to the mitotically active spermatogonia, a marked contrast in response is evident.

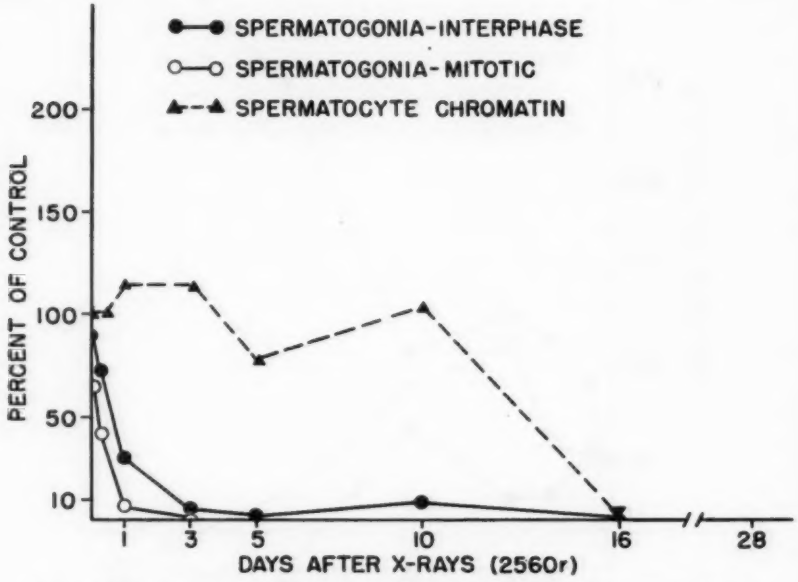

FIGURE 3. Strain S. Incidence of spermatogonia and spermatocytes at different times following 2560 r of x-rays.

Thus by one hour after irradiation the area occupied by this class has declined to 65% of the control in the case of strain S and 30% of the control in strain Ba. In both strains at one day after exposure, the area has further declined to less than 10% of the control value. Thereafter no spermatogonial mitotic activity was recorded for strain S during the remainder of the experiment. In the case of strain Ba, the response is essentially the same except that a low level of spermatogonial activity (4% of control) was encountered in the 10-day material.

Two other cell classes disappear from the seminiferous epithelium following exposure to 2560 r of x-rays. These are the spermatocytes and spermatids. The respective areas occupied by these cells have declined to zero levels by 16 days after exposure.

In view of the recorded differences in behavior of the sperm fraction of the two strains (see Table I, and Figures 2 and 4), it is unfortunate that 28-day material from strain S was not available. The response over the period of 1–10 days is rather similar. Over the period 10–28 days, strain Ba data indicate a progressive decline to a very low level at 28 days whereas strain S, in contrast, shows a marked increase during the 10–16-day period.

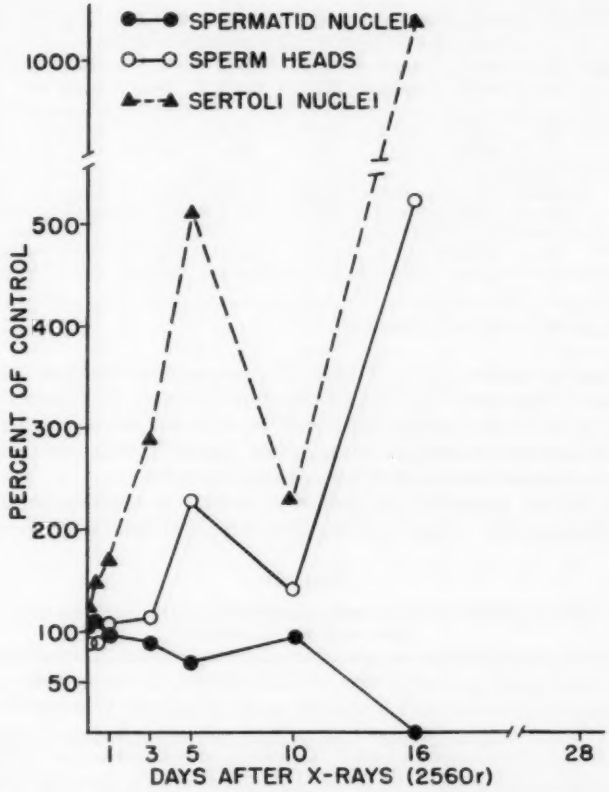

FIGURE 4. Strain S. Incidence of spermatids, sperm and Sertoli nuclei at different times following 2560 r of x-rays.

The data pertaining to the Sertoli cell fraction indicate that the trend is similar in both strains but differs in magnitude. Thus in the case of strain Ba the relative area occupied by this class undergoes a steady increase over the period 0–5 days at which time the level reached is about 2.5 times the control. This value then falls to about 2 times the control value by 10 days, and then increases again reaching at 16 days a level about 17 times the control value. In the case of strain S

TABLE I

Frequency of cell types at various times after 2560 r of x-rays[2]

Stage and strain		1 hour	8 hours	1 day	3 days	5 days	10 days	16 days	28 days
Spermatogonia in interphase	Ba	103.5	72.5	51.6	13.4	7.7	13.3	0.0	0.0
	S	90.1	73.5	29.9	5.5	1.1	8.9	0.0	—
Spermatogonia in mitosis	Ba	30.5	9.7	1.7	5.1	0.0	4.3	0.0	0.0
	S	65.4	41.9	6.9	0.0	0.0	0.0	0.0	—
Spermatocyte chromatin	Ba	105.6	111.4	95.1	128.5	99.2	49.7	0.0	0.0
	S	100.9	100.0	115.1	114.8	79.0	104.0	0.0	—
Spermatid nuclei	Ba	113.5	105.6	110.7	72.4	68.1	138.1	0.0	0.0
	S	106.2	110.2	97.8	91.6	69.7	97.7	0.0	—
Sperm heads	Ba	59.9	88.9	106.3	120.8	222.2	178.2	78.4	4.4
	S	89.8	91.2	107.3	113.5	227.5	139.7	521.6	—
Sertoli nuclei	Ba	97.9	107.6	181.6	223.2	250.1	212.5	1,702	1,860
	S	121.6	143.6	172.1	293.4	510.5	227.2	1,036	—

[2] Values expressed as per cent of controls.

the corresponding values are at 5 days 5 times, and at 10 days 2.2 times the control levels. The value attained by 16 days is about 11.4 times the control level. The cause of this striking difference between the strains, at 5 days, is not clear. These changes in relative areas of the Sertoli fraction are, within limits, reflections of changes in area of the spermatogenic cells.

In Table II are presented the data with respect to spermatogonial and non-spermatogonial necrosis. Data from our previous experiment (320 r) are included

TABLE II

Spermatogonial and non-spermatogonial necrosis at various times after 320 r or 2560 r of x-rays

Strain and time after x-rays		320 r		2560 r	
		% spermatogonial necrosis	% non-spermatogonial necrosis	% spermatogonial necrosis	% non-spermatogonial necrosis
Ba	Control	3.8	2.1	3.8	2.1
S		4.0	2.5	4.0	2.5
Ba	1 hour	3.4	3.0	6.0	3.7
S		5.8	2.4	6.5	1.2
Ba	8 hours	9.7	2.2	15.3	3.0
S		8.8	1.7	14.9	2.0
Ba	24 hours	7.4	2.3	0.0	4.5
S		4.6	2.2	0.0	3.2

in this table for purposes of comparison. All data in this table are expressed as percentages.

Changes in area occupied by the spermatogonial fraction (normal interphasic + mitotic + necrotic spermatogonia) following exposure to x-rays are presented in summary form in Table III.

TABLE III

A comparison of expected and observed frequencies of spermatogonia at various times following exposure to x-rays of different dose levels

Time after x-rays	Strain					
	Ba			S		
	% total spermatogonia	Difference from control	Difference as % of control	% total spermatogonia	Difference from control	Difference as % of control
Control	12.35			15.10		
320 r 1 hour	14.47	+2.12	17.17	13.55	−1.55	10.26
8 hours	9.89	−2.46	19.92	12.29	−2.81	18.61
24 hours	10.39	−1.96	15.87	6.78	−8.32	55.10
2560 r 1 hour	9.11	−3.24	26.23	13.21	−1.79	11.85
8 hours	10.27	−2.08	16.84	11.61	−3.49	23.11
24 hours	3.32	−9.03	73.12	3.01	−12.09	80.07

DISCUSSION

In the case of the testis it is clear that any treatment which interferes with, or prevents, spermatogonial mitotic activity will bring about partial or complete maturation depletion of the seminiferous tubules. Temporary depletion will follow if, for a short period of time, the level of spermatogonia is reduced much below normal thereby preventing the quantitative replacement of the spermatocyte fraction. Such a reduction may be brought about either by inhibition of chromosomal reduplication (and therefore of mitosis), a relatively high level of spermatogonial necrosis or by some combination of these. Depletion of the permanent type may be brought about in the same manner but with the added proviso that spermatogonial regeneration must also be prevented.

There is ample evidence on hand that exposure to x-rays brings about the onset of maturation depletion (see introduction for references). It then follows that the effects of a dose of x-rays large enough to produce a permanent absence of spermatogonia must differ quantitatively and/or qualitatively from the effects of a dose causing only temporary changes. The present work is concerned with

the response of the mouse testis to a dose of x-rays large enough to induce permanent depletion of the seminiferous tubules.

In order to facilitate comparison with the present results, a brief resumé of previous results utilizing a dose of x-rays of 320 r (Bryan and Gowen, 1956) is included here. With respect to interphasic spermatogonia, our 320 r data indicate a marked decline to less than 10% of the control value by three days following exposure. This low level remains in effect until 10 days, at which time regeneration commences. Spermatogonial mitotic activity follows a different course. There is an initial decline reaching a low point 8 hours after exposure, then a marked rise during the 8–24-hour period. This is followed by a further and more extensive decline during 1–3 days post-irradiation. Thereafter the pattern of response is essentially the same as for the non-dividing cells. The 2560 r data show marked deviations from this pattern. The area occupied by interphasic spermatogonia undergoes a rapid decline reaching a very low level by 5 days. There is a slight rise during the 5–10-day period, but the level then declines to zero by 16 days. The mitotic spermatogonia follow a similar pattern but the rate of decline during the early post-irradiation period (1–24 hours) is much more rapid. There is, then, no rise in mitotic activity during the 8–24-hour period; nor does repopulation of the seminiferous tubules take place. These facts lend themselves to the interpretation, that following exposure to 2560 r of x-rays, spermatogonial mitosis must be delayed or inhibited for a longer period of time than in the case of the 320 r experiment. Furthermore the surviving spermatogonia must be unable to sustain a regenerative phase after the initial inhibitory effects of the irradiation have worn off.

As stated above, spermatogonial necrosis may contribute to some extent to the depletion process. It is also to be expected that large doses of x-rays would be likely to produce a greater frequency of cell death than small doses. Hence it is probable that spermatogonial cell death may play a more prominent role in the initiation of maturation depletion following exposure to large doses of x-rays. With these points in mind, the pertinent sections were analyzed (by the Chalkley method) for spermatogonial and non-spermatogonial necrosis. These data are listed in Table II together with corresponding data obtained from the 320 r experiment. Exposure to either dose of x-rays increases the frequency of necrosis above control levels by one hour after irradiation. Similarly, a peak is reached at the 8-hour period followed by a return to lower levels at 24 hours. The pattern of response is therefore about the same for either dose of x-rays; however, there is a difference in the magnitude of this response. At 8 hours after irradiation with 2560 r, the frequency of spermatogonial necrosis is almost double that found following exposure to 320 r. Then at 24 hours following exposure no cells were encountered which could be classified as necrotic spermatogonia. These observations may be interpreted in two ways. On the one hand it may mean that a large fraction of cells are heavily damaged and undergo degenerative changes even though they are far removed, in time, from mitotic activity. The remaining cells, then, constitute a less severely damaged fraction which may undergo degeneration with the onset of mitosis. This interpretation is in accord with the views of Lasnitski (1943) concerning the effect of large doses of x-rays (2500–10,000 r). The possibility also exists that the observed absence of necrotic spermatogonia is correlated

with the very low mitotic rate 24 hours following exposure. This view receives some support from the reports of Glücksmann and Spear (1939) and others, to the effect that irradiation-induced cellular degeneration occurs at about the same time as the onset of mitotic activity. Then it follows that under conditions where mitotic levels are low (as in the present case) the chances of encountering necrotic cells are likewise much reduced. It is obvious from the present data that spermatogonial nuclei survive for different periods of time (some are present at 5–10 days following exposure). This must mean that some cells have suffered less damage than others, yet this fraction also is destined to be eliminated from the tubules by 16 days following x-ray exposure. From this we may conclude that severely damaged cells may undergo degeneration prior to the onset of mitosis (in agreement with Lasnitski), while less severely damaged cells do not degenerate until they attempt to enter mitosis.

With respect to the foregoing discussion, the relations between the levels of necrosis induced by different doses of x-rays are of significance. As Table II shows, an 8-fold increase in dose approximately doubles the frequency of necrotic cells at the 8-hour period. Although the conditions of the present experiments are quite different from those of Lasnitski (1943), who used tissue cultures of chick fibroblasts, nevertheless the results are fairly similar. This author's results indicate that a four-fold increase in dose increased the frequency of necrosis to about 1.4 times that of the low dose, whereas a 1.1-times increase in necrosis resulted when the dose was doubled. If a curve is fitted to these data it is found that an 8-fold increase in dose should result in the approximate doubling of the necrotic level (as observed in the present work). This offers further support for the idea that, as the x-ray dose is increased, cells further removed from the sensitive period are likely to suffer lethal injury.

It may be argued that fixed and stained preparations do not allow a very accurate estimation of the frequency of necrosis. Unless the intervals between the fixation times are less than the time necessary for cells to undergo lysis and be eliminated, such estimates are likely to be minimum values. However this is open to verification. Thus from control data the total area of the tubules occupied by spermatogonia (normal + necrotic) can be determined. A comparison of this value with similar determinations on irradiated material will reveal the goodness of fit existing between the expected and observed frequencies. The important point is whether or not any irradiation-induced decrease in area occupied by normal spermatogonia can be accounted for by an increase in the necrotic value. An analysis of this kind is summarized in Table III. It can be seen that, with the exception of the Ba 320 r, one-hour material, each time period shows a deficiency of spermatogonia. Following exposure to 320 r, these deficiencies range from about 10% to 20% of control values during the first 8 hours. With respect to the 2560 r experiment, the corresponding values range from about 12% to 26% of controls. It is clear that these changes in area are, during the first 8 hours, quite similar despite the difference in dose levels employed. Since the data in Table III take into account spermatogonial necrosis, the observed deficiencies cannot be accounted for on the basis of the increased frequency of necrosis as reported in Table II.

On *a priori* grounds it would be logical to impute the observed difference to

additional and "unobserved" spermatogonial necrosis. This is not an entirely satisfactory explanation. The stage at which cells are most sensitive to irradiation corresponds to that portion of the mitotic cycle during which chromosomal reduplication is taking place. Irradiation does not inhibit DNA synthesis in cells which are already in the period of synthesis, but delays these cells in entering division (see Howard and Pelc, 1953). At the time of irradiation, then, there is a fraction of spermatogonia which has passed the critical stage. These cells are most probably those observed in mitosis during the first few hours following exposure. In confirmation of this are the results of Bullough and Van Oordt (1950) and others, which indicate that, in the mouse, the duration of mitosis (prophase–telophase) is of the order of three hours. Now a certain proportion of these dividing spermatogonia transform into spermatocytes. These products of division are, therefore, lost from the spermatogonial fraction. Thus it follows that shortly after irradiation, the spermatogonial fraction will be decreased both by loss of these cells and by the reduction in frequency of replacement divisions. Unfortunately it is not possible to calculate the decrease in the spermatogonial fraction to be expected on these grounds. However it is evident that the deficiencies reported in Table III cannot entirely be ascribed to "unobserved" necrosis. The estimates of necrosis as determined in the present work are, therefore, reliable indices of irradiation-induced cellular degeneration.

The effects described above are cumulative, and therefore any deficiency should become progressively more marked with time following exposure to large doses of x-rays. Reference to Table III shows that this is the case. Exposure to 2560 r results in reduction of spermatogonial area to 20–27% of control values by 24 hours. In addition, it was expected that the 2560 r data would show trends similar to those following exposure to 320 r, but of greater magnitude. The data of Tables II and III are in agreement with this, but the relation is somewhat obscured by variation in the level of spermatogonia scored at 24 hours following exposure to 320 r. Since relatively few animals were used in these experiments, sampling errors undoubtedly contribute to this observed variation between strains.

In the case of the 320 r experiment, the surviving fraction of spermatogonia eventually repopulate the tubules. This suggests that recovery has occurred prior to the onset of mitotic activity 10 days following exposure. It is also possible to interpret these findings to mean that the surviving spermatogonia constitute a relatively more resistant fraction. Such an interpretation would be in accord with the conclusions of Eschenbrenner *et al.* (1948). In marked contrast are the results of the 2560 r experiment. Here, also, a small fraction of spermatogonia are present at 5 days following exposure. At 10 days, both strains show a slight increase in spermatogonia over the 5-day levels, but by 16 days, spermatogonia have been completely eliminated. These observations may be explained by the assumption of a further period of necrosis during the 10–16-day interval. This implies, in contrast to the 320 r case, that the spermatogonia present at 5 days subsequently attempt to undergo mitosis at which time latent damage expresses itself.

The spermatogonia present at 5 days following exposure to either dose of x-rays have the morphological characteristics of the so-called type A or "dusty" spermatogonia. Type A cells are regarded as "stem-line" germ cells by Clermont and Leblond (1953). These authors point out that a small fraction of Type A

spermatogonia after one division cycle become "dormant." Such "dormant" cells do not divide again until later in the spermatogenic cycle. In other words these spermatogonia remain "dormant" for about 6 days following the initiation of this inactive phase. This means that, in these cells, recovery from the effects of radiation exposure should be possible before mitosis recommences. With respect to our 320 r material, this apparently is the case. On the other hand, any recovery following exposure to 2560 r must only be partial since repopulation does not occur —despite the suggestion of an increase in frequency of spermatogonia by 10 days post-irradiation. On these grounds the spermatogonia present at 5 days following exposure to 2560 r (some or all of which may in fact be "dormant" type A cells) must be capable of limited division. Otherwise, the frequency of spermatogonia would not undergo the changes observed during the 5–16 day period.

Further evidence which points up the more widespread damage produced by exposure to this high dose of x-rays is provided by a consideration of non-spermatogonial necrosis. Reference to Table II shows that at 24 hours, the necrotic level has risen to 1.5–2.0 times that of the control. This increase can be largely accounted for on the basis of the very high frequency of degenerating metaphase I spermatocytes. It was observed that practically all metaphase I plates present in sections of these tubules were necrotic. In the 320 r experiment, the frequency of non-spermatogonial necrosis remained close to control levels. Very few necrotic metaphase I spermatocytes were encountered in this material.

The present data, when considered together with the results of our 320 r experiment, allow several conclusions to be drawn with respect to the manner in which the histological effects of radiation exposure are brought about. Certain of these conclusions take on added significance when considered in the light of other experimental approaches. Cells are prevented from entering division following irradiation. This may come about either through inhibition of DNA synthesis (chromosomal reduplication) or by death of the cells. Both mechanisms play a role in the irradiation-induced depletion of spermatogonia. The death of cells plays a more important role in this process following high doses of irradiation—such as 2560 r—than following exposure to low doses (320 r in the present case). Irradiation-induced mitotic inhibition would appear to be the major factor following exposure to relatively low doses of x-rays. As is readily apparent, this effect of x-rays on cells is of a very basic nature. It must perforce be taken into consideration if the nature of the effects of irradiation on biological systems is to be evaluated in a proper manner.

It is clearly evident that different levels of injury are produced by the irradiation treatments used here. Thus following 320 r, the surviving spermatogonial fraction is capable of mitotic activity to the extent necessary for the initiation of tubule repopulation. After exposure to 2560 r, on the other hand, the survivors are incapable of such a sustained effort.

Summary

1. Changes in the cellular composition of the seminiferous tubules induced by exposure to 2560 r of x-rays have been analyzed by a quantitative histological procedure. These data have been compared with the results obtained following ex-

posure to a much lower dose (320 r) in an attempt to gain further insight with respect to the manner in which the observed changes are brought about.

2. Exposure to 320 r results in a temporary maturation depletion of the seminiferous epithelium. This is brought about mainly by the inhibition of spermatogonial mitosis with irradiation-induced spermatogonial necrosis playing only a minor role. In contrast, exposure to 2560 r produces a permanent depletion due to the fact that surviving spermatogonia are incapable of sustained regenerative efforts.

3. The frequency of necrotic spermatogonia, following 2560 r, was found to be double the peak value attained in the 320 r material, or four times that of the corresponding controls.

4. Taken together, the data for the 320 r and 2560 r experiments suggest that spermatogonial depletion is brought about in two ways: (1) by suppression of mitosis due to inhibition of DNA synthesis, and (2) the killing of cells. Irradiation-induced necrosis plays a much more important role following exposure to high doses of x-rays. Even so the frequency of necrosis in either experiment did not reach very high levels, being about 9% after the low dose and about 15% in the case of the high x-ray dose.

5. Further evidence was obtained in support of the view that relatively heavily damaged cells may undergo degenerative changes prior to the onset of division, while less heavily damaged cells manifest degenerative changes only at about the time of entry into mitosis.

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LARVAL DEVELOPMENT OF BALANUS AMPHITRITE VAR. DENTICULATA BROCH REARED IN THE LABORATORY[1]

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While there have been numerous studies on Cirripedia larvae based on reconstructed life-histories, to date there have been only three reports on nauplii reared from the egg through all larval stages to the sessile form, that of Herz (1933) for *Balanus crenatus,* Hudinaga and Kasahara (1941) for *Balanus amphitrite hawaiiensis,* and Costlow and Bookhout (1957) for *Balanus eburneus.* This method has the advantage over reconstructed life-histories in that one can be sure of the identity of the adult, the source of eggs and future larvae. Once the life-histories of all species of barnacles in a given area have been described, ecological studies may be made with a greater degree of assurance. Ecological investigations based on sampling, such as that of Bousfield (1955), may provide the number of stages, the approximate duration and mortality of the individual stages, and the distribution and fluctuations in large populations. Laboratory studies on individually reared larvae, however, can give more detailed information on all phases of the life-history other than distribution and population fluctuations. Both types of research are required before a complete picture can be obtained. Laboratory studies are necessarily prerequisite to physiological and genetic investigations.

Balanus amphitrite denticulata Broch is one of the most widely distributed acorn barnacles. It has been reported from the tidal waters of Britain which are artificially heated by industrial effluents (Crisp and Molesworth, 1951), the estuaries of South Africa (Sandison, 1954), and the East and West coasts of North America (Dr. Dora Henry, personal communications). In spite of its world-wide distribution only the first two larval stages have been described (Sandison, 1954). Bishop (1950) believes that *Balanus amphitrite denticulata* merits a more thorough study and questions its position as a variety of *B. amphitrite.* Thus it should be of interest to compare the larval development of this variety with the descriptions of larvae of *Balinus amphitrite albicostatus* (Ishida and Yasugi, 1937) and *Balanus amphitrite hawaiiensis* (Hudinaga and Kasahara, 1941).

Balanus amphitrite denticulata is the most abundant fouling organism in the inter-tidal region at Beaufort, North Carolina and breeds during the same summer months as *Balanus eburneus.* During the past two years we have followed the larval development of *B. amphitrite denticulata* in the laboratory to determine the number of stages, the frequency of molting, and the duration of the intermolt periods. Our secondary objectives were to compare the appendage setation and body form with the corresponding naupliar stages of the other two varieties of *Balanus amphitrite* which have been described and with the larvae of barnacles belonging to different species and genera.

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The technique of rearing individual larvae of *Balanus amphitrite denticulata* was identical to that described for *Balanus eburneus* (Costlow and Bookhout, 1957). Mass cultures of the larvae were maintained on a diet of *Chlamydomonas* sp. and fertilized *Arbacia* eggs at a constant temperature of 26° C.

Results and Discussion

The nauplii of *Balanus amphitrite denticulata* reared individually in the laboratory pass through 6 stages and one cyprid stage.

Nauplii. The most significant morphological characteristics of each naupliar stage are given below.

Stage I. (Fig. 1, I.) The curved frontolateral horns project caudo-laterally, are relatively long, and situated close to the carapace. The carapace is rounded and the abdominal process terminates in two short spines (Fig. 1, Ia). All setae are devoid of setules (Fig. 1, Ib, c, d).

Stage II. (Fig. 1, II.) The frontolateral horns project laterally. Anterior to the small lateral spines the carapace has relatively straight lateral borders. Posterior to the spines the carapace tapers into the caudal process which bears a small dorsal and ventral tooth at its midpoint (Fig. 1, IIa). The abdominal process bears one spine on each side of the base and the maxillules are prominent (Fig. 1, IIa). The majority of the setae now bear setules (Fig. 1, IIb, c, d).

Stage III. (Fig. 1, III.) The frontolateral horns are tapered from a slightly swollen basal portion adjoining the carapace. The lateral edges of the carapace are rounded and the lateral spines of stage II are missing. The abdominal process bears the same spines as in stage II (Fig. 1, IIa).

Stage IV. (Fig. 2, IV.) A pair of short carapace spines marks the posterior edge where the carapace is delimited from the caudal process. Two pairs of spines are located on the abdominal process. The posterior pair is located at the base of the abdominal process and the anterior pair is in line with the division between the caudal and abdominal processes. A pair of small teeth appears just anterior to the bifurcated end of the abdominal process. Six rows of minute bristles are found on the ventral surface of the abdominal process (Fig. 2, IVa).

Stage V. (Fig. 2, V.) The anterior and posterior pairs of spines of the abdominal process remain as in stage IV. Lateral to the posterior abdominal spine, however, a smaller spine is added on each side. The sub-terminal teeth and bristles remain as in the previous stage (Fig. 2, Va).

Stage VI. (Fig. 2, VI.) Six pairs of small spines on the ventral surface of the abdominal process replace the bristles of the previous stage. The developing cirriform appendages are visible *beneath* the exoskeleton, but are not shown in Figure 2, VIa. The anterior pairs of spines, found in stage V, are not present but the small paired lateral spines and large paired median spines remain. Paired eyespots develop in the later sixth stage nauplii.

Cyprid. The rounded anterior end of the cyprid is the widest portion of the carapace, curving gradually to the posterior end. The degree of pigmentation varies considerably but some brown pigment was always observed. When the bivalve carapace is closed both the anterior and posterior ends are smooth.

The number of naupliar stages observed for *B. amphitrite denticulata* is consistent with our findings for *Balanus eburneus* (Costlow and Bookhout, 1957) and

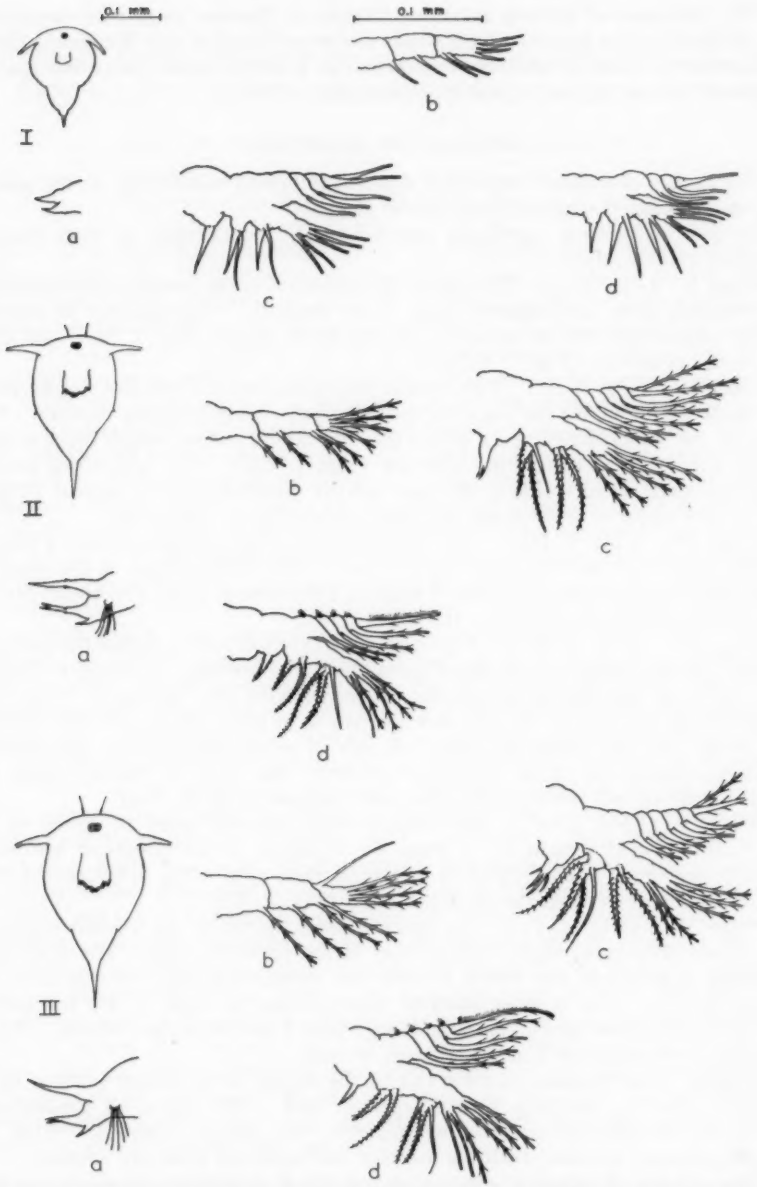

FIGURE 1. Carapace, caudal and abdominal processes, and appendages of naupliar stages I, II, and III of *Balanus amphitrite denticulata* reared in the laboratory. All swimming setae are cut short. a, lateral view of abdominal and caudal processes; b, antennule; c, antenna; d, mandible.

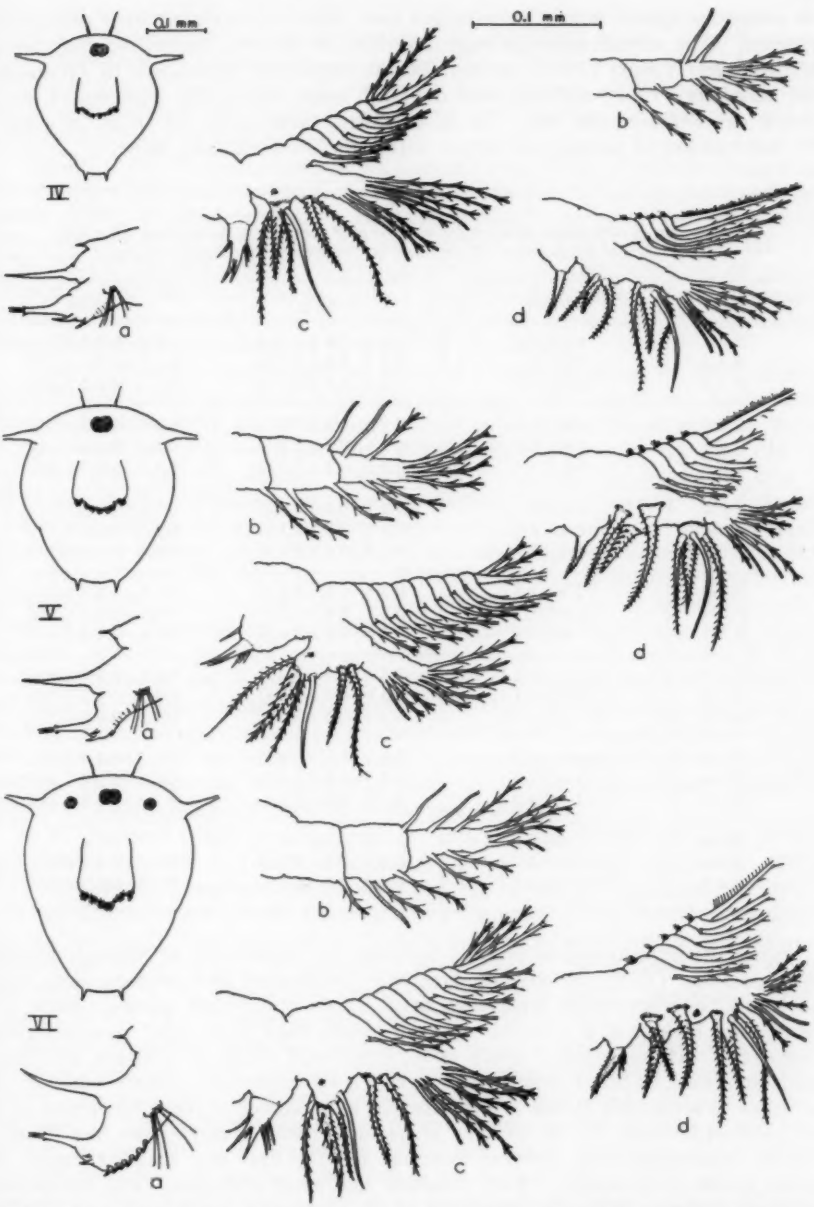

FIGURE 2. Carapace, caudal and abdominal processes, and appendages of naupliar stages IV, V, and VI of *Balanus amphitrite denticulata* reared in the laboratory. All swimming setae are cut short. a, lateral view of abdominal and caudal processes; b, antennule; c, antenna; d, mandible.

for numerous species whose larval stages have been reconstructed from planktonic material. The seventh naupliar stage described for *Balanus amphitrite albicostatus* by Ishida and Yasugi (1937) and for *Balanus amphitrite hawaiiensis* by Hudinaga and Kasahara (1941) differed from the sixth stage only in the presence of completely developed paired eyes. In *B. amphitrite denticulata*, as in *B. eburneus*, the development of paired eyes occurs within the sixth naupliar stage.

TABLE I

Comparison of appendage setation for nauplii of B. amphitrite denticulata (B.a.d.), B. amphitrite albicostatus (B.a.a.) and B. amphitrite hawaiiensis (B.a.h.)

Stage		Antennule	Antenna	Mandible
I	*B.a.d.*	0.4.2.1.1.	0.2.3.–0.3.2.2.2.G.	0.1.3.–0.3.2.2.2.G.
	B.a.a.	0.4.2.1.–	0.1.4.–0.3.2.2.2.G.	0.1.3.–0.3.2.3.2.G.
	B.a.h.	0.4.2.1.1.	0.2.3.–0.3.2.2.3.G.	0.1.3.–0.3.2.3.3.G.
II	*B.a.d.*	0.4.2.1.1.	0.2.5.–0.3.2.2.3.G.	0.1.4.–0.3.2.3.2.G.
	B.a.a.	0.4.2.1.1.	0.1.6.–0.3.2.2.2.G.	0.1.3.–0.3.2.3.2.G.
	B.a.h.	0.4.2.1.1.	0.2.5.–0.3.2.2.3.G.	0.1.4.–0.3.2.3.3.G.
III	*B.a.d.*	1.4.2.1.1.	0.2.5.–0.3.2.2.4.G.	0.1.4.–0.3.3.3.2.G.
	B.a.a.	1.4.2.1.1.	0.1.6.–0.3.2.2.3.G.	0.1.3.–0.4.2.3.2.G.
	B.a.h.	1.4.2.1.1.	0.2.5.–0.3.2.2.4.G.	0.1.4.–0.3.3.3.3.G.
IV	*B.a.d.*	1.1.4.2.1.1.	0.3.6.–0.5.3.2.4.G.	0.1.4.–0.4.3.4.3.G.
	B.a.a.	1.1.4.2.1.1.	0.2.7.–0.4.3.2.3.G.	0.1.4.–0.4.2.3.3.G.
	B.a.h.	1.1.4.2.1.1.	0.3.6.–0.5.3.2.4.G.	0.1.4.–0.4.3.3.3.G.
V	*B.a.d.*	1.1.1.4.2.1.1.1.	0.3.8.–0.5.3.2.4.G.	0.1.5.–0.4.4.4.3.G.
	B.a.a.	1.1.1.4.2.1.1.1.	0.2.9.–0.4.3.2.3.G.	0.1.5.–0.4.2.3.3.G.
	B.a.h.	1.1.1.4.2.1.1.1.	0.3.8.–0.5.3.2.4.G.	0.1.5.–0.4.4.4.3.G.
VI	*B.a.d.*	1.1.1.4.2.1.2.1.	0.4.8.–0.5.3.2.4.G.	0.1.5.–0.4.4.4.3.G.
	B.a.a.	1.1.1.4.2.1.2.1.	0.3.9.–0.5.3.2.4.G.	0.1.5.–0.4.2.4.4.G.
	B.a.h.	1.1.1.4.2.1.2.1.	0.3.9.–0.5.3.2.4.G.	0.1.5.–0.4.4.4.3.G.
VII	*B.a.d.*	none	none	none
	B.a.a.	1.1.1.4.2.1.2.1.	0.3.9.–0.5.3.2.4.G.	0.1.5.–0.4.2.4.4.G.
	B.a.h.	1.1.1.4.2.1.2.1.	0.3.9.–0.5.3.2.4.G.	0.1.5.–0.4.4.4.3.G.

Since the introduction of setation formulae for appendages of barnacle nauplii by Bassindale (1936) all investigators have maintained that setation alone was not a reliable criterion for separating larval stages of different species. Some investigators believe it is a useful criterion when used with other morphological characters, such as length of whole body, length and width of carapace, and body spine structure. Others believe it is merely a developmental feature which is of no value in separating larvae. Nevertheless, most authors have continued to give the setation formulae for the naupliar stages described. Knight-Jones and Waugh (1949) summarized the setation formulae for the first two naupliar stages of seven species of barnacles. They conclude that small differences and similarities in setation during the early stages are of no systematic significance and may be

used only as secondary criteria for identification of stages of a given species. In order to evaluate whether setation formulae are significant or not, even as secondary characters, we believe it would be helpful to first determine if the formulae of closely related varieties of *B. amphitrite* are identical or different. If they prove to be different, and useful in separating varieties, they may well be used to separate species as well as genera.

Table I gives the appendage setation for each stage of *B. amphitrite denticulata* and compares this variety with *B. amphitrite hawaiiensis* and *B. amphitrite albicostatus*. The antennule setation is identical for each corresponding stage of all three varieties but can be used to determine whether one is examining stage III, IV, V or VI. Using the setation of the antenna and mandible, however, all stages of the three varieties other than the fifth of *B. amphitrite hawaiiensis* and *B. amphitrite denticulata* can be separated from one another with the setation of the mandible being more consistently different than that of the antenna.

Table II gives the other species of *Balanus*, as well as species of other genera, which have the same setation of individual appendages as *B. amphitrite denticulata*. Setation formulae from the available literature indicate that when all three pairs of appendages are considered, the first stage of *B. amphitrite denticulata* can be separated from all other species described. For example, *B. amphitrite albicostatus* has identical antennule and antenna setations but the mandible is different (Table II). Stage II of *B. amphitrite denticulata* is identical in setation of all appendages to *Balanus improvisus*, *Chthamalus dentatus*, and *Octomeris angulosa* as shown in Table II by the appearance of these three species in all three columns. While the third stage may be separated from all other species by setation, the fourth stage of *B. amphitrite denticulata* is identical to the fourth stage of *Balanus trigonus*. The fifth naupliar stage has setation identical to *B. amphitrite hawaiiensis*, *B. eburneus*, and *B. improvisus*, while the sixth stage nauplius is identical only to *B. improvisus* (Jones and Crisp, 1954). Thus, in areas where two or more of these species of barnacles are associated and there is an overlapping of the breeding season, setation could not be relied upon as the only criterion for identification. It is significant, however, that after the second naupliar stage, identical setation of all appendages is confined to the genus *Balanus*.

In *B. eburneus* (Costlow and Bookhout, 1957), as in *Balanus crenatus* (Pyefinch, 1949) the setation of each individual stage was found to be consistent. This was true also of *B. amphitrite denticulata* and the variability described by Norris *et al.* (1951) for second stage *Balanus improvisus* and *B. amphitrite denticulata* was not observed. Sandison (1954) gives the setation formulae for the appendages of the first two stages of *B. amphitrite denticulata*. Our findings differ from Sandison's only in the antennary exopodite of the second stage. Sandison (1954) gives 0.2.5—0.3.2.2.2.G. whereas we found 0.2.5—0.3.2.2.3.G.

Another character which has proved to be useful when combined with other criteria is carapace width and length and total length, although the latter is less reliable. In cases where setation is identical, marked differences in carapace dimensions may be useful in identification. When dimensions are similar or overlap, however, as they do for *B. amphitrite denticulata* and *B. eburneus* (Table III), this character cannot be used. Both of these species have been reared on *Chlamydomonas* sp. and *Arbacia* eggs at 26° C. in a continuously lighted culture cabinet.

TABLE II

Setation of B. amphitrite denticulata nauplii and other species which have identical setation in one or more appendages

Stage	Antennule	Antenna	Mandible
I	0.4.2.1.1. *B. a. denticulata* *B. a. albicostatus* *B. a. hawaiiensis* *B. algicola* *B. balanoides* *B. crenatus* *B. eburneus* *B. improvisus* *B. perforatus** *B. trigonus* *C. dentatus* *E. modestus* *M. mitella* *O. angulosa* *T. serrata* *T. squamosa* *V. stroemia*	0.2.3.–0.3.2.2.2.G. *B. a. denticulata* *B. a. albicostatus*	0.1.3.–0.3.2.2.2.G. *B. a. denticulata* *B. algicola* *B. crenatus* *B. eburneus* *B. improvisus* *B. perforatus* *B. trigonus* *C. dentatus* *C. stellatus* *E. modestus* *M. mitella* *O. angulosa* *T. serrata* *T. squamosa* *V. stroemia*
II	0.4.2.1.1. *B. a. denticulata* *B. a. albicostatus* *B. a. hawaiiensis* *B. algicola* *B. balanoides* *B. crenatus* *B. eburneus* *B. improvisus* *B. perforatus* *B. trigonus* *C. dentatus* *C. stellatus* *E. modestus* *M. mitella* *O. angulosa* *T. serrata* *T. squamosa* *V. stroemia*	0.2.5.–0.3.2.2.3.G. *B. a. denticulata* *B. a. hawaiiensis* *B. improvisus* *B. perforatus* *B. trigonus* *C. dentatus* *O. angulosa*	0.1.4.–0.3.2.3.2.G. *B. a. denticulata* *B. algicola* *B. improvisus* *C. dentatus* *O. angulosa* *T. serrata*
III	1.4.2.1.1. *B. a. denticulata* *B. a. hawaiiensis* *B. a. albicostatus* *B. algicola* *B. eburneus* *B. improvisus* *B. perforatus* *B. trigonus* *C. stellatus* *E. modestus* *M. mitella* *O. angulosa* *T. squamosa* *V. stroemia*	0.2.5.–0.3.2.2.4.G. *B. a. denticulata* *B. a. hawaiiensis* *B. improvisus* *B. perforatus* *B. trigonus* *O. angulosa*	0.1.4.–0.3.3.3.2.G. *B. a. denticulata*

TABLE II—*Continued*

Stage	Antennule	Antenna	Mandible
IV	1.1.4.2.1.1. *B. a. denticulata* *B. a. albicostatus* *B. a. hawaiiensis* *B. algicola* *B. balanoides* *B. eburneus* *B. improvisus* *B. perforatus* *B. trigonus* *C. stellatus* *E. modestus* *M. mitella* *O. angulosa* *T. squamosa*	0.3.6.–0.5.3.2.4.G. *B. a. denticulata* *B. a. hawaiiensis* *B. trigonus*	0.1.4.–0.4.3.4.3.G. *B. a. denticulata* *B. improvisus* *B. trigonus*
V	1.1.1.4.2.1.1.1. *B. a. denticulata* *B. a. albicostatus* *B. a. hawaiiensis* *B. algicola* *B. eburneus* *B. improvisus* *B. perforatus* *B. trigonus* *O. angulosa*	0.3.8.–0.5.3.2.4.G. *B. a. denticulata* *B. a. hawaiiensis* *B. eburneus* *B. improvisus* *E. modestus*	0.1.5.–0.4.4.4.3.G. *B. a. denticulata* *B. a. hawaiiensis* *B. eburneus* *B. improvisus* *B. perforatus* *E. modestus*
VI	1.1.1.4.2.1.2.1. *B. a. denticulata* *B. a. albicostatus* *B. a. hawaiiensis* *B. algicola* *B. crenatus* *B. eburneus* *B. improvisus* *B. trigonus* *B. perforatus* *O. angulosa*	0.4.8.–0.5.3.2.4.G. *B. a. denticulata* *B. eburneus* *B. improvisus* *E. modestus*	0.1.5.–0.4.4.4.3.G. *B. a. denticulata* *B. a. hawaiiensis* *B. improvisus* *B. perforatus* *B. trigonus* *E. modestus*

* Norris and Crisp, 1954. All other sources indicated in text.

B—Balanus
C—Chthamalus
E—Elminius
M—Mitella
O—Octomeris
T—Tetraclita
V—Verruca

We have no personal observations on how these larvae compare in size with those from the natural environment. The first two stages of *B. amphitrite denticulata* reared by us at Beaufort are slightly larger than those described by Sandison (1954) from South Africa which in turn were larger than those found by Crisp (Sandison, 1954) in Great Britain.

Body form and spine structure should also be considered with setation and body dimensions. The general body form of all nauplii of the three varieties of

Balanus amphitrite is similar but there are differences in spine structure. The lateral spines of the second stage of *B. amphitrite denticulata* (Fig. 1, II), observed also by Sandison (1954), are not found in *B. amphitrite albicostatus* or *B. amphitrite hawaiiensis*. Ishida and Yasugi (1937) show six pairs of small spines on the ventral surface of the abdominal process in the third stage of *B. amphitrite albicostatus*. These do not appear in *B. amphitrite denticulata* or in *B. eburneus* (Costlow and Bookhout, 1957) until the sixth naupliar stage.

Earlier workers introduced into general usage the term "metanauplius," apparently referring to the sixth stage barnacle nauplius. Inasmuch as additional functional appendages are not added during the sixth naupliar stage, it cannot correctly be identified as a metanauplius. It is true that the six pairs of cirriform appendages appear during the sixth stage but they are underneath the exoskeleton and do not function until the cyprid stage. In the larval Cirripedia described to date no true metanaupliar stage has been described.

TABLE III

Measurements of larval stages of B. amphitrite denticulata (B.a.d.) and B. eburneus (B.e.) reared under laboratory conditions at 26° C.

Stage	Carapace				Total length (mm.)	
	Width (mm.)		Length (mm.)		*B.a.d.*	*B.e.*
	B.a.d.	*B.e.*	*B.a.d.*	*B.e.*		
I	0.14–0.20	0.16–0.18	—	—	0.16–0.24	0.19–0.23
II	0.19–0.24	0.21–0.23	—	—	0.25–0.30	0.32–0.34
III	0.22–0.27	0.23–0.27	—	—	0.30–0.34	0.35–0.38
IV	0.25–0.32	0.27–0.32	0.25–0.32	0.30–0.33	0.35–0.39	0.40–0.42
V	0.29–0.36	0.29–0.34	0.30–0.35	0.35–0.38	0.41–0.47	0.44–0.48
VI	0.35–0.43	0.36–0.39	0.35–0.40	0.42–0.50	0.47–0.53	0.54–0.60
C	—	—	—	—	0.49	0.46

The duration of individual stages of *B. amphitrite denticulata* was similar to that found for *B. eburneus* at 26° C. The first stage was short, as in many other species, and lasted from ten minutes to six hours. The duration of the second stage was one day and that of the third stage, one to three days with an average of 1.5 days. Stage IV lasted from one to two days with an average of one day. Stage V averages 1.5 days and stage VI, 2.5 days but with greater variation than in the earlier stages. The molting frequency was similar to that observed for *B. eburneus* (Costlow and Bookhout, 1957).

The size range of *B. amphitrite denticulata* cyprids, 0.45 mm.–0.53 mm., overlaps the range of *B. eburneus* cyprids. Doochin (1951) reported an average length of 0.53 mm. for cyprids of *B. amphitrite niveus* and notes that because of the variation in length it could not safely be used to distinguish cyprids *of B. amphitrite niveus* from those of *B. improvisus*. Barnes (1953) found "at least" two distinct modes in size frequency curves for *B. balanoides* and *B. crenatus* cyprids and interprets them as two populations of distinct sizes, developing in different environments. Pyefinch (1948), noting the discrepancy in *Balanus balanoides* cyprid

sizes as reported by Runnström (1925) and Bassindale (1936), suggests that the cyprid attains a greater length in more northern waters. Pyefinch (1948) uses pigmentation and position of eyes to separate the cyprids of *B. balanoides* and *B. crenatus*. Unfortunately, these characters are similar in *B. amphitrite denticulata* and *B. eburneus* cyprids. In both species the anterior end is broader and the dorsal surface curves back to the posterior end. Both posterior and anterior ends are smooth when the bivalve carapace is closed, as in *B. amphitrite niveus*, and the notched appearance of *B. improvisus* cyprids (Doochin, 1951) has not been observed. No definite external characteristic has been found in this study which may be relied upon to differentiate cyprids of *B. amphitrite denticulata* and *B. eburneus*.

The cyprid stage of *B. amphitrite denticulata* could be maintained in the laboratory from one to eight days but successful attachment was observed only in those which settled one to three days following the final naupliar molt. Hudinaga and Kasahara (1941) noted that the settling time of *B. amphitrite hawaiiensis* varied considerably, ranging from a few hours to five days after the final naupliar ecdysis.

The over-all time of development at 26° C. of *B. amphitrite denticulata* from hatching to the settled stage, ranged from seven to ten days, the same time interval observed for *B. eburneus* (Costlow and Bookhout, 1957), when reared at the same temperature. Hudinaga and Kasahara (1941) found that *B. amphitrite hawaiiensis* required seven days at 23–28° C. to attain the cyprid stage and usually one additional day to settle and metamorphose. Ishida and Yasugi (1937) observed that *B. amphitrite albicostatus* attained the cyprid stage in approximately two weeks at 20–25° C. None of their cyprids settled and metamorphosed. Hudinaga and Kasahara (1941) note that *B. amphitrite albicostatus* required only one week for complete development to the young barnacle when fed enough *Skeletonema costatum*. Yasugi (1937) reported that the goose barnacle, *Mitella mitella* L., required nine days for development to the cyprid at 26–28° C. but at 23–26° C. the period was extended to twelve days.

Mortality in *B. amphitrite denticulata* was higher than that of *B. eburneus*. In *B. eburneus* the greatest mortality occurred in the fifth and sixth stages. In *B. amphitrite denticulata* there was no mortality in the fifth stage, 22.2 per cent in the sixth stage, and 49.2 per cent in the cyprid.

While Hudinaga and Kasahara (1941) do not give the approximate number of either *B. amphitrite hawaiiensis* or *B. amphitrite albicostatus* that successfully settled, they do point out that if the food was not adequate, the time for development to the cyprid stage was increased and that survival was reduced. Survival of *B. amphitrite denticulata* under laboratory condition was 12.7 per cent.

Summary and Conclusions

From a study of 126 isolated *Balanus amphitrite denticulata* nauplii reared in the laboratory, plus hundreds in mass culture, the following conclusions may be drawn:

1. The free-swimming larvae of *Balanus amphitrite denticulata* consist of six naupliar stages and one cyprid stage.

2. Carapace size, appendage setation, and spine structure are given for each naupliar stage.

3. Most naupliar stages of *Balanus amphitrite denticulata* may be separated from the larvae of *B. amphitrite albicostatus* and *B. amphitrite hawaiiensis* on the basis of setation formulae; the fifth stage of *Balanus amphitrite denticulata* and *Balanus amphitrite hawaiinensis* have identical setation of all appendages.

4. A comparison of appendage setation with larvae of other species and genera of barnacles described to date indicates that setation formulae are not definitive in distinguishing all stages of all species. Setation is, however, a valuable criterion for identification of nauplii and may be used to separate certain stages and species.

5. At 26° C., the duration of the six naupliar stages of *Balanus amphitrite denticulata* is as follows: first stage, ten minutes to six hours; second stage, one day; third stage, one to three days with an average of 1.5 days; fourth stage, one to two days with an average of one day; fifth stage, one to three days with an average of 1.5 days; and the sixth stage, one to five days with an average of 2.5 days.

6. The duration of the cyprid stage ranges from one to eight days. Successful settling and metamorphosis were observed only in those which settled one to three days after the final naupliar molt.

7. The time required for complete larval development in the laboratory at 26° C. was seven to ten days after hatching. Successful settling and metamorphosis were observed in 12.7 per cent of the 126 nauplii studied under segregated laboratory conditions.

8. Mortality, under laboratory conditions, was highest during the sixth naupliar stage (22.2 per cent) and the cyprid stage (49.2 per cent).

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SURVIVAL AND GROWTH OF CLAM AND OYSTER LARVAE AT DIFFERENT SALINITIES

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Adult clams (*Venus mercenaria*) and oysters (*Crassostrea virginica*) are found, both in areas where the salinity is almost oceanic, and in areas where it is low. There is little published information on the effects of salinity on the reproductive processes of the hard clam. Turner and George (1955) reported an experiment in which early larvae of *V. mercenaria* were introduced into the bottom of a glass tube in which layers of sea water of diminishing salinity were placed one above the other. The larvae swam upward, through the sharp gradients that separated the layers, with no loss in velocity until they had passed the boundary between the sea water at 20.0 parts per thousand and that at 15.0 p.p.t. In the latter their velocity decreased and they no longer moved upward. Instead, they swam in a circular pattern just above the interface. Turner, in a personal communication, reported rearing clam larvae to metamorphosis at salinities of 31.0, 28.0, 24.0 and 20.0 p.p.t. He reports, however, that he had a constant mortality in 20.0 p.p.t. until, by the tenth day, he had only about 20 per cent as many living larvae at this salinity as were still living at the higher salinities.

Since Korringa (1941) has reviewed the literature relating to the effects of salinity on several species of oysters, only those works dealing with American oysters will be mentioned here. Ryder (1885), Nelson (1921), Hopkins (1931), Loosanoff (1932) and other investigators have attempted to evaluate, from field data, some of the effects of salinity on various phases of oyster physiology. In addition, Loosanoff (1948, 1952) found experimentally that adult Long Island Sound oysters developed functional spermatozoa and fertilizable eggs at a salinity of 7.5 p.p.t. but that these eggs did not develop normally. In lower salinities gonad development was arrested. He found, however, in one experiment that Long Island Sound oysters, which were already ripe, spawned at salinities as low as 5.0 p.p.t. Butler (1949), in a study of oysters from upper Chesapeake Bay, concluded that gametogenesis was inhibited in 90 per cent of the surviving population until salinity levels rose to about 6.0 p.p.t.

Amemiya (1926) and Clark (1935) studied the salinity range for the development of fertilized eggs, of the American oyster, into shelled larvae. Both concluded that 14.5 or 15.0 p.p.t. was the lower limit for normal development and that 39.0 p.p.t was the upper limit. Amemiya, however, believed the optimum salinity for development was from 25.0 to 29.0 p.p.t., whereas Clark thought the optimum was at 23.0 p.p.t.

Nelson (1921), in New Jersey waters, observed active free-swimming larvae in salinities ranging from 5.17 to 28.80 p.p.t. From this and his observation that the adult oyster closed and refused to feed at salinities below 10.42 p.p.t.,

Nelson concluded that oyster larvae (p. 38) "may become accustomed to much lower densities than the adult animal will stand, and still remain active."

Prytherch (1934) studied the salinity limits for the attachment and metamorphosis of oyster larvae and found that they could attach in salinities from 5.6 to 32.2 p.p.t., but that beyond these limits (p. 71) "no setting occurred though many of the larvae crawled for periods of over four hours." Moreover, although (p. 71) "setting was accomplished with considerable regularity in salinities ranging from 9.0 to 29.0 p.p.t.," beyond these limits only a small percentage of the larvae was able to complete the process. He believed that the salinity range from 16.0 to 18.0 p.p.t. was optimum for setting.

In the present study we have re-investigated the effect of salinity on development of fertilized eggs of the American oyster, *C. virginica,* into shelled larvae to obtain quantitative data for estimating the relative percentage of eggs developing normally in different salinities. Similar studies were also made on eggs of the hard clam, *V. mercenaria.* In addition, we determined the effects of several lowered salinities on the survival and growth of free-swimming larvae of both clams and oysters after they had reached the straight-hinge stage.

Development of Fertilized Eggs at Different Salinities

Methods

Our laboratory tap water cannot be used, without treatment, to lower the salinity because it contains enough metallic ions, chiefly copper, to be toxic to developing eggs. In Experiment No. 1 we did use tap water to lower the salinity but added a chelator to bind up the excess metal ions. In Experiment No. 2 we used distilled water to dilute our usual sea water. This water was from a Stokes still that discharged into a tin-lined storage tank. In all subsequent experiments a Barnstead BD-2 demineralizer was our source of salt-free water, and this water was stored either in Pyrex carboys or polyethylene tanks.

High salinity water was obtained by evaporation of our sea water in polyethylene containers until the salinity was 44.52 p.p.t. This water was then stored in a Pyrex carboy until used in these experiments. The salinities tested in Experiment No. 3 were obtained by making appropriate dilutions of this high salinity water with our usual sea water (27.0 p.p.t.). In Experiment No. 4 all the salinities were obtained by diluting the high salinity water with demineralized water. The salinities tested in Experiment No. 5 were obtained by diluting our usual sea water with demineralized water.

The animals used in these experiments were spawned in the usual manner (Loosanoff and Davis, 1950; Davis, 1953). Fertilized eggs of both oysters and clams were thus obtained free of the body tissues and excessive sperm that may have affected the results of earlier workers, who used stripped eggs and sperm. For each experiment eggs from several females were pooled and an equal number of eggs was taken from this mixed lot to start cultures at each of the different salinities. All containers were then covered, to prevent loss of water by evaporation, and placed in the constant temperature bath at 23.0° C. Forty-eight hours later the contents of each culture vessel were screened and the number of normal straight-hinge larvae determined. Experimental errors, including transfer of the

eggs to the experimental culture vessels, recovery of the larvae and sampling, can probably account for differences of not more than ± 10 per cent in any individual experiment.

Salinity tolerance of developing eggs of V. mercenaria

Eggs of the hard clam of Long Island Sound can develop into normal straight-hinge larvae only within the relatively narrow salinity range of 20.0 to 32.5 p.p.t.

TABLE I

Comparison of the percentage of eggs, of oysters and clams, that develops to normal straight-hinge larvae in sea water of different salinities. Highest number developing to straight-hinge larvae from each spawning taken as 100 per cent. Experiments No. 3, No. 4 and No. 5 from same spawning

Salinity (in p.p.t.)	Eggs of Long Island Sound oysters		Eggs of Peconic Bay oysters			Eggs of Long Island Sound clams		
	Exp. No. 1	Exp. No. 2	Exp. No. 3	Exp. No. 4	Exp. No. 5	Exp. No. 3	Exp. No. 4	Exp. No. 5
44.5				0				
40.0				0				
35.0			56	29		0	1.0	
32.5			99	35		52	34	
30.0			92	78		54	84	
27.5				75			100	
Control* (26.0–27.0)	89	87	88	88	92	92	92	100
25.0				85			91	
22.5	100	100		94	100		80	84
20.0	90	82		84	76		21	16
17.5	79	91		58	63		0	0
15.0	64	73			48			0
12.5	<0.1	<0.1			18			0
10.0	0	0			0			0
7.5	0	0			0			0
5.0					0			0
2.5					0			0

* Control—Milford Laboratory sea water.

(Table I). At a salinity of 35.0 p.p.t. only one per cent or less of the eggs developed into shelled larvae, and at 17.5 p.p.t. none of the eggs developed into normal shelled larvae. Even at 20.0 p.p.t. only 16 to 21 per cent of the eggs developed into straight-hinge larvae and at 32.5 p.p.t. only 34 to 52 per cent reached this stage. Thus, the salinity range for practical work, extending from 22.5 to 30.0 p.p.t., is narrower than the biological or absolute range (20.0 to 32.5 p.p.t.). In our experiments, the optimum salinity for the development of clam eggs was about 26.5 to 27.5 p.p.t.

Salinity tolerance of developing eggs of C. virginica

Some eggs of Long Island Sound oysters, conditioned at 26.0 to 27.0 p.p.t., developed into normal straight-hinge larvae in salinities as low as 12.5 p.p.t. (Experiments No. 1 and No. 2, Table I, and Experiment No. 6, Table II). The highest percentage of normal straight-hinge larvae was obtained at a salinity of 22.5 p.p.t. The percentage of normal larvae obtained in salinities below 22.5 p.p.t. decreased progressively, in most experiments, with each successive decrease in salinity down to 15.0 p.p.t. Below 15.0 p.p.t. the percentage of normal larvae decreased abruptly and in a salinity of 12.5 p.p.t. less than 0.1 per cent of the eggs developed normally.

TABLE II

Comparison of the relative percentage of normal straight-hinge larvae obtained at salinities (a) from eggs of oysters from different areas at the same salinity and (b) from eggs of oysters from the same area at different salinities

	Oysters conditioned and spawned at 26.0–27.0 p.p.t.				Hodges Bar oysters that developed gonads at about 8.74 p.p.t.*		
					Oysters spawned in		
	Long Island Sound Exp. No. 6	Peconic Bay Exp. No. 7	Hodges Bar Exp. No. 6	Hodges Bar Exp. No. 7	7.5 p.p.t. Exp. No. 8	10.0 p.p.t. Exp. No. 8	15.0 p.p.t. Exp. No. 8
Control 26.0–27.0 p.p.t.	100	100	82	91	0	0	0
25.0 p.p.t.	99	86	80	94	0	0	0
22.5 p.p.t.	100	87	90	100	0	0	26
20.0 p.p.t.	100	86	100	91	7	26	72
17.5 p.p.t.	92	76	87	81	50	76	92
15.0 p.p.t.	37	58	60	45	48	100	100
12.5 p.p.t.	<0.1	13	1.4	11	83	92	89
10.0 p.p.t.	0	0	0	0	100	98	78
7.5 p.p.t.	0	0	0	0	99	96	72
5.0 p.p.t.	0	0	0	0	0	0	0
2.5 p.p.t.	0	0	0	0	0	0	0

* These oysters were kept, for four days prior to spawning, at the salinities at which they were induced to spawn.

Eggs of oysters from Peconic Bay, where the salinity may be as high as 31.0 p.p.t., showed about the same percentage developing in each of the lower salinities as did eggs of Long Island Sound oysters, except that a slightly higher percentage of these eggs developed at a salinity of 12.5 p.p.t. (Experiments No. 4 and No. 5, Table I, and Experiment No. 7, Table II).

In salinities above 22.5 p.p.t. the percentage of normal larvae again decreased progressively with each successive increase in salinity up to 35.0 p.p.t. Because of the comparatively high percentage of eggs that developed normally in 35.0 p.p.t., we suspect that at least a few eggs would have developed in 37.5 p.p.t. (not tested), although in 40.0 p.p.t. none developed normally.

The salinity tolerance of eggs of Maryland oysters from Hodges Bar, a low salinity area, differed only slightly from that of eggs of Long Island Sound oysters when both groups of parent oysters were conditioned at 26.0–27.0 p.p.t. (Experi-

ments No. 6 and No. 7, Table II). However, when Maryland oysters from the same area developed gonads in their native habitat, where the salinity was only 8.74 p.p.t. at the time they were collected, and were spawned in salinities of 7.5, 10.0 and 15.0 p.p.t., the eggs developed into normal straight-hinge larvae at 10.0 p.p.t., and larvae, normal in shape and only slightly smaller in size, developed at 7.5 p.p.t. (Experiment No. 8, Table II). Even at 5.0 p.p.t. many of the eggs developed into very early shelled stages before they died. The upper salinity limit for development of normal larvae from eggs of oysters which developed gonads and were spawned at low salinities was also appreciably lower than for eggs of oysters from the same area that developed gonads and were spawned at 26.0–27.0 p.p.t. None of the eggs produced at low salinities developed into normal larvae at salinities above 22.5 p.p.t.

Effect of Lowered Salinities on Growth of Larvae

Methods

To determine the effect of lowered salinities on growth of larvae we started with straight-hinge clam and oyster larvae 48 hours old. These larvae were obtained by spawning clams and oysters in sea water at our normal salinity (26.0–27.0 p.p.t.). Several 18-liter cultures of the eggs of each species were then set up in sea water at that salinity and permitted to develop for 48 hours. At the end of this period the larvae from all the cultures were collected on stainless steel screens to give a single combined culture of clam larvae and one of oyster larvae. The number of larvae per ml. in each combined culture was determined and appropriate volumes used to start duplicate cultures of clam larvae and duplicate cultures of oyster larvae at each of the salinities tested.

In both experiments the water in which the larvae were kept was changed every second day. Since the food used was grown in sea water of our normal salinity, it was added before the salinity was adjusted after a change of water. In the first experiment additional food was given to the cultures on the days between changes, as was our usual practice. The salinity could not be adjusted after this additional food was given, however, and it was found that this increased the salinity of the cultures appreciably. The increase in salinity, while only about 0.5 p.p.t. in cultures in which the nominal salinity was 22.5 p.p.t., was as much as 1.5 p.p.t. in cultures nominally at 10.0 p.p.t. and lower.

In the second experiment the salinities were maintained at the nominal level. About 1½ times the usual amount of food was given on the days when the water was changed and the salinity adjusted, but no additional food was given until the next change of water.

Effect on clam larvae

The results of the two experiments on clam larvae were in general agreement in that growth of larvae was comparatively good at salinities of 20.0 p.p.t. and higher (Fig. 1). In both experiments larvae were reared to metamorphosis at these salinities. In the first experiment there was no appreciable difference in growth of the larvae at 20.0 p.p.t., 22.5 p.p.t. and at our normal salinity (26.0–27.0 p.p.t.). However, in the second experiment, in which the salinities were controlled more care-

fully, the rate of growth of larvae decreased progressively at each successively lower salinity, as shown by the average size at each measuring period. At a salinity of 17.5 p.p.t., although growth of the clam larvae was significantly slower than at normal salinity, some larvae did reach metamorphosis. These larvae were sluggish and, apparently, more susceptible to disease than were larvae kept at higher salinities. Thus, even though some larvae reached metamorphosis, such a high mortality occurred during and immediately after setting that we were unable to follow their growth further.

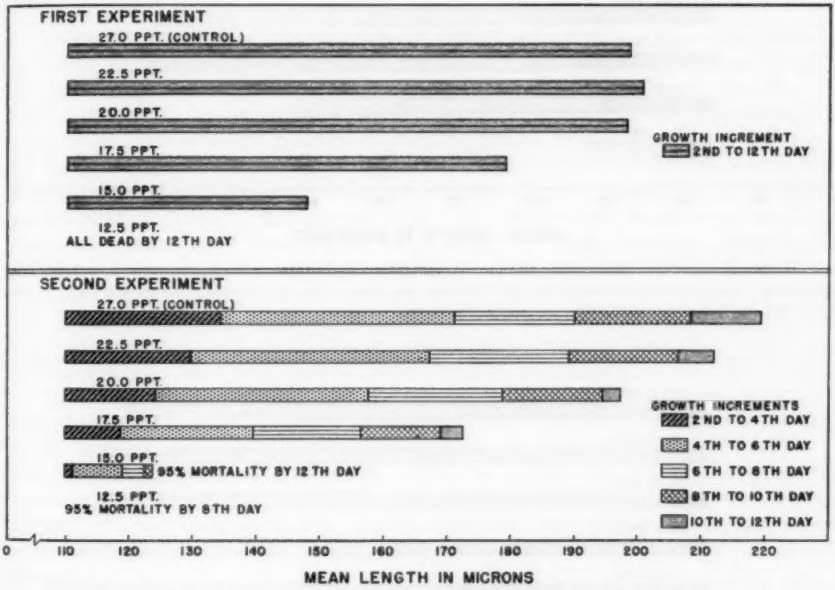

FIGURE 1. Growth of clam larvae at different salinities. Samples were taken and measurements made only at the beginning and termination of the first experiment. In the second experiment, salinities were more carefully controlled and samples, from each of the duplicate cultures at each salinity, were taken every second day. One hundred larvae from each sample were measured.

Clam larvae kept at a salinity of 15.0 p.p.t. grew even more slowly than those kept at 17.5 p.p.t. They were sluggish and susceptible to attack by protozoa, fungus and bacteria. In each experiment some larvae lived more than 12 days, but all died before reaching setting size.

At a salinity of 12.5 p.p.t., a few clam larvae survived for 10 or 12 days, but did not grow. In the second experiment, when samples were taken every two days, a slight but progressive decrease in size of larvae kept at this salinity was noted. In both experiments, it was observed that at salinities of 12.5 p.p.t. and lower, the shells of dead clam larvae were completely disintegrated in approximately 48 hours. The progressive decrease in size of larvae at 12.5 p.p.t., therefore, suggests that the shells, even of living larvae, were being slowly dissolved.

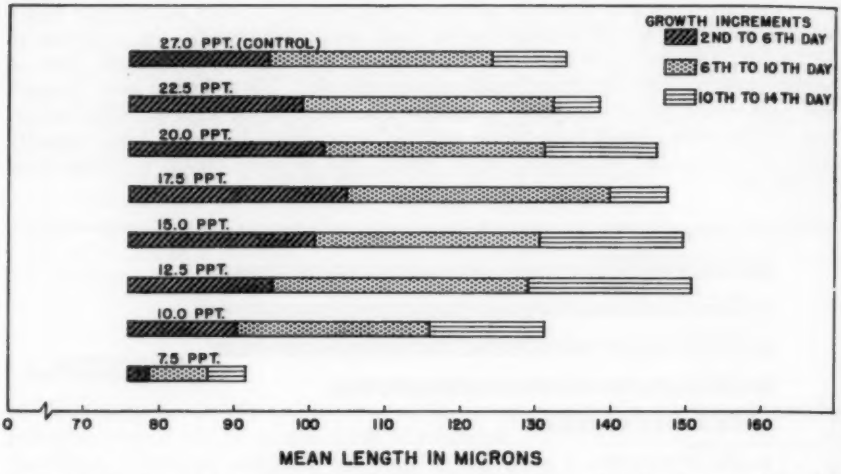

FIGURE 2. Growth of oyster larvae at different salinities. Samples, from each of the duplicate cultures at each salinity, were taken on the sixth, tenth and fourteenth days. One hundred larvae from each sample were measured.

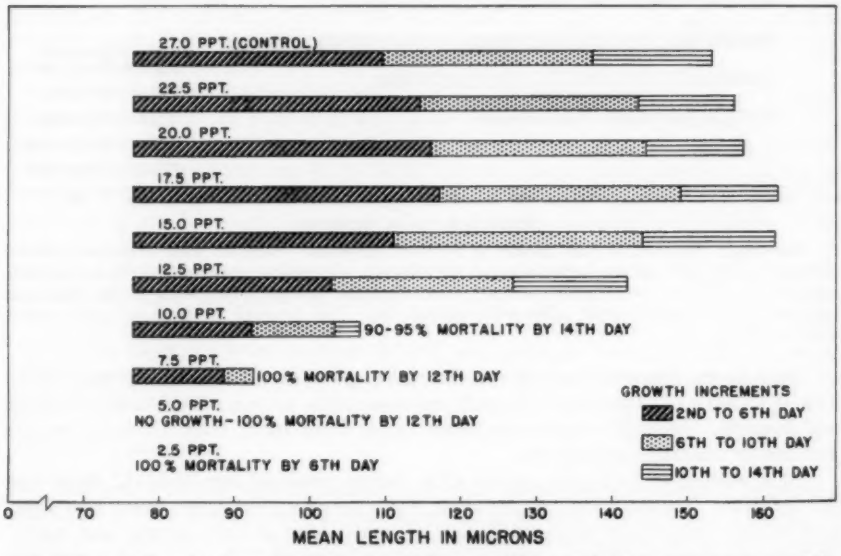

FIGURE 3. Growth of oyster larvae at different salinities. Salinities were more carefully controlled than in the previous experiment. Samples from each of the duplicate cultures at each salinity, were taken on the sixth, tenth and fourteenth days. One hundred larvae from each sample were measured.

At salinities of 10.0 p.p.t. or lower, clam larvae showed no growth and all were dead within six days.

The lower borderline salinity for clam larvae appears to be about 17.5 p.p.t. Clam larvae and set would probably survive and grow slowly at this salinity if all other conditions were nearly ideal, but would probably die if some other environmental factor were unfavorable. It would seem exceedingly doubtful that conditions in nature would ever be so favorable that clam larvae could survive, reach setting stage, and continue to grow at salinities of 15.0 p.p.t. or lower.

Effect on oyster larvae

Oyster larvae grew at a comparatively normal rate in all salinities down to and including 12.5 p.p.t. However, a salinity of 17.5 p.p.t. was optimum, by a slight margin, for growth of larvae at least through the tenth day (Figs. 2 and 3). In both experiments larvae kept in a salinity of 15.0 p.p.t. had, by the fourteenth day, attained an average length equal to or slightly greater than those kept at 17.5 p.p.t. In the first experiment the larvae kept at 12.5 p.p.t. were, by the fourteenth day, slightly larger than those at any other salinity, and even the larvae kept at 10.0 p.p.t. were almost as large as the controls (Fig. 2). In the first experiment, however, due to the addition of food on the days when the water was not changed, the salinities of these cultures actually ranged from 12.5 to about 14.0 p.p.t. and from 10.0 to 11.5 p.p.t., respectively. In the second and other experiments, in which the salinity was more carefully controlled, growth of larvae at 12.5 p.p.t. was appreciably slower than at 15.0 p.p.t., while larvae kept at 10.0 p.p.t. grew considerably slower than the controls, and in some experiments mortality was high.

In the first experiment, in which the salinity of the cultures nominally at 7.5 p.p.t. actually ranged from 7.5 to 9.0 p.p.t., some oyster larvae lived through the 14 days of the experiment, although growth was slow and mortality high (Fig. 2). In the second experiment, and others in which the salinity was held at 7.5 p.p.t., the larvae appeared to feed and seemed quite normal for the first few days, even though growth was very slow (Fig. 3). By the eighth to tenth day at this salinity, however, oyster larvae appeared moribund and by the twelfth day mortality was almost complete.

At a salinity of 5.0 p.p.t. oyster larvae appeared moribund within 48 hours. After four days almost all were completely dead, and in only a few could ciliary motion be detected.

Thus far, we have the results of only one experiment using larvae of oysters from low salinity areas in Maryland. While the results of a single experiment are not always reliable, this experiment indicated that when these oysters are conditioned at a salinity of 26.0–27.0 p.p.t. their larvae do not tolerate any lower salinities than do larvae from Long Island Sound oysters conditioned at the same salinity. As a matter of fact, in this experiment the optimum growth of the Maryland larvae was at 22.5 p.p.t., and the rate of growth decreased progressively with each successive decrease in salinity below this optimum.

Effect on older oyster larvae

The results of the above experiments, in which we started with straight-hinge larvae, could be interpreted as indicating that larger larvae (10 to 14 days old)

can grow as fast as or faster, at a salinity of 15.0 p.p.t. (or even 12.5 p.p.t. in the first experiment), than at 17.5 p.p.t. We believed, however, that this was because the larvae at 17.5 p.p.t., being larger at 10 days, were more severely handicapped by insufficient food. Davis and Guillard (in press) have shown that it requires approximately four times the quantity of food given these cultures to maintain maximal growth of larvae after they reach an average length of 140 μ, yet such a quantity of food would have handicapped the smaller or slower growing larvae at the other experimental salinities.

To test the above hypothesis in another experiment we determined the rate of growth of older larvae at salinities of 26.0–27.0 p.p.t. (control), 17.5, 15.0, 12.5, 10.0 and 7.5 p.p.t. A culture of oyster larvae, that had been reared to a mean length of 165 μ at normal salinity, was divided into six smaller cultures and one

TABLE III

Comparison of growth of older oyster larvae at different salinities. Measurements are in microns

	Control 26.0–27.0 p.p.t.	17.5 p.p.t.	15.0 p.p.t.	12.5 p.p.t.	10.0 p.p.t.	7.5 p.p.t.
Initial mean length	165.55	165.55	165.55	165.55	165.55	165.55
Length after 8 days at different salinities	206.30	214.00	203.05	205.75	186.80	175.49
Increase	40.75	48.45	37.50	40.20	21.25	9.94

of these was kept at each of the above salinities. Eight days later it was found that the larvae kept at 17.5 p.p.t. had grown the fastest, while there was very little difference in size between the controls and those kept at 15.0 or 12.5 p.p.t. (Table III). The larvae kept at 10.0 p.p.t., however, had increased in size only about one-half as much as the controls, and those kept at 7.5 p.p.t. had increased only about one-fourth as much as the controls.

These cultures were continued for several more days to get an indication of the setting rate of larvae in the different salinities. Those kept in 17.5 p.p.t. gave significantly more spat than any of the other cultures, but setting was also good at 15.0 and 12.5 p.p.t. A few spat were obtained at 10.0 p.p.t. but the larvae kept at 7.5 p.p.t. all died before metamorphosis.

DISCUSSION

Our experiments on the development of fertilized clam eggs indicate that even with a salinity as high as 22.5 p.p.t., the reproductive potential of clams may be reduced as much as 15 to 20 per cent. If the salinity is reduced to 20.0 p.p.t., the reproductive potential of clams is reduced 80 to 85 per cent and if the salinity over the spawning beds should be as low as 17.5 p.p.t., there appears to be no possibility of obtaining normal larvae.

Once clam larvae have attained the straight-hinge stage, we find, as did Turner (personal communication), that the larvae grow quite well at 20.0 p.p.t. but contrary to Turner's results we find no significant mortality at this salinity. Turner and George's (1955) observation that the larvae swam upward, the normal reaction, until they came into the sea water at 15.0 p.p.t. also appears significant. We find that at both 17.5 and 15.0 p.p.t. the larvae appear sluggish, grow slowly and suffer high mortality either prior to reaching setting stage (15.0 p.p.t.) or during metamorphosis (17.5 p.p.t.).

The minimum salinity at which a good percentage of clam eggs develops into straight-hinge larvae is 22.5 p.p.t. Once the larvae have attained this stage, however, they survive and grow well at a salinity as low as 20.0 p.p.t. Thus, as Loosanoff, Miller and Smith (1951) showed for the temperature requirements of eggs and larvae of *V. mercenaria*, the embryonic stages cannot tolerate as wide a range of salinities as can the larval stages. Similarly, Chanley (in press) found that small juvenile clams (1.8 to 3.6 mm. in length) survived for a month or more at 15.0 p.p.t. but died in salinities of 12.5 p.p.t. or lower, while larger juveniles (5.0 to 21.5 mm. in length) survived at 12.5 p.p.t.

Much additional research is needed to find the minimum salinities at which adult clams develop gonads, to find whether the salinity at which the parents develop gonads influences the salinity tolerance of the eggs and larvae, and to find whether races tolerant of low salinities exist or can be developed.

By comparison with the status of research on clams, the relation of salinity to the reproductive processes of oysters appears to be fairly well documented. Thus, the findings of Loosanoff (1948, 1952) and Butler (1949) appear to agree quite well that 6.0 to 7.5 p.p.t. is the minimum salinity for the development of gametes by the American oyster. Additional research is required, however, to determine more clearly the value of gametes produced at low salinities.

In general, our results on the development of fertilized eggs of oysters conditioned at a salinity of 27.0 p.p.t. are in close agreement with those of Amemiya (1926) and Clark (1935). However, possibly because of improvements in technique, use of larger cultures and repeated trials, or due to differences in the salinities at which the oysters developed gonads, we have been able to demonstrate that a few eggs of such oysters will develop normally at 12.5 p.p.t. or about 2.5 p.p.t. lower than found by earlier workers.

The very close agreement of the results of Amemiya (1926), Clark (1935), and ours with oysters conditioned at 26.0–27.0 p.p.t., researches so widely separated in time, space and populations sampled, suggested that the salinity tolerance of eggs of the American oyster was quite similar throughout the range of the mollusk. Preliminary tests with Maryland oysters from Hodges Bar, a low salinity area, that had been artificially conditioned at 27.0 p.p.t., appeared to confirm this suggestion. Additional research, however, showed that this was not true when these oysters developed gonads in their native habitat where the salinity was only 8.74 p.p.t. at the time the oysters were collected. When these oysters were spawned at salinities of 7.5, 10.0 or 15.0 p.p.t., comparatively normal larvae were obtained in a salinity as low as 7.5 p.p.t. It may be that eggs of oysters developing gonads at even lower salinities can develop at salinities below 7.5 p.p.t.

Additional research is needed to determine the value of larvae developing at

such low salinities. Will such larvae survive, grow and set? Will they grow and set at lower salinities than larvae developing at higher salinities?

Our observation that, once they have reached the straight-hinge stage, oyster larvae may live for some time at salinities as low as 5.0 p.p.t. suggests that the larvae Nelson (1921) reported swimming at a salinity of 5.17 p.p.t., and those observed by many other workers at low salinities, may simply be survivors of larval populations accidentally carried into these low salinities. Our observations, even of older larvae, at 10.0 p.p.t., indicate that growth at this salinity is extremely slow although a very few of the larvae kept at this salinity did set. Perhaps larvae from oysters that develop gonads at low salinities survive better and grow faster at salinities of 10.0 p.p.t. and lower than do larvae from oysters conditioned at 26.0–27.0 p.p.t. Otherwise, our observations on growth, coupled with Prytherch's (1934) observations on the setting of larvae at different salinities, would seem to suggest that oyster sets, occurring in areas where the salinity is only 10.0 p.p.t. or lower, are dependent upon larvae that grow almost to setting size at higher salinities and are carried to low salinity setting areas as practically fully mature larvae. Moreover, Chanley's results (in press) indicate that recently set spat, like the larvae, grow best at salinities near 17.5 p.p.t. and that growth is significantly retarded by salinities of 10.0 p.p.t. or lower. Unlike the larvae some of his spat grew slowly at a salinity of 5.0 p.p.t. although only about 40 per cent survived at this salinity.

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Summary

1. The optimum salinity for the development of straight-hinge larvae from eggs of clams from Long Island Sound is about 27.5 p.p.t.

2. The salinity range for development of eggs of these clams is from 20.0 p.p.t., at which salinity only 16 per cent to 21 per cent of the eggs develop, to 35.0 p.p.t., at which salinity only 1 per cent or less of the eggs develops normally.

3. The optimum salinity for growth of clam larvae after they reach the straight-hinge stage is 27.5 p.p.t. or higher, while 15.0 p.p.t. is the lowest salinity at which appreciable growth occurs, and 17.5 p.p.t. is the lowest at which we were successful in rearing clam larvae to metamorphosis.

4. Both the optimum salinity and the salinity range for the development of straight-hinge larvae from eggs of the American oyster appear to be governed by the salinity at which the parent oysters develop gonads.

5. The optimum salinity for the development of eggs of oysters from Long Island Sound, Peconic Bay, and Hodges Bar, Maryland was about 22.5 p.p.t. when these oysters developed gonads at a salinity of 26.0–27.0 p.p.t.

6. When Hodges Bar oysters developed gonads at a salinity of approximately 8.74 p.p.t. the optimum salinity for the development of their eggs was between

10.0 and 15.0 p.p.t. and appeared to be dependent upon the salinity at which the parent oysters were kept immediately prior to spawning.

7. The salinity range for development of normal straight-hinge larvae from eggs of these low salinity oysters was from 7.5 to 22.5 p.p.t., whereas the range for eggs from oysters conditioned at 26.0–27.0 p.p.t. was from 12.5 to above 35.0 p.p.t.

8. The optimum salinity for growth of larvae of Long Island Sound oysters, conditioned and spawned at 26.0–27.0 p.p.t., was 17.5 p.p.t.

9. The optimum salinity for growth of larvae of Hodges Bar oysters, conditioned and spawned at 26.0–27.0 p.p.t., appeared to be about 22.5 p.p.t.

10. It is still undetermined whether the optimum salinity for growth of larvae is influenced by the salinity at which the parent oysters develop gonads.

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RESPIRATION IN TISSUES OF GOLDFISH ADAPTED TO HIGH AND LOW TEMPERATURES

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Poikilothermic animals adapted to low temperatures are very different physiologically from warm-adapted animals. These differences have been detected in terms of lethal temperatures, metabolic variations, and other gross changes. In recent years an interest has arisen in the cellular mechanisms of these changes.

Peiss and Field (1950) measured the oxygen consumption of minced brains and sliced livers from cold- (0°–1° C.) adapted cod (*Boreogadus saida*) and from warm- (25° C.) adapted golden orfe (*Idus melanotus*). Differences in Q_{O_2} of both liver and brain were in the same direction as for intact animals. Liver and brain excised from the cold-adapted animals consumed oxygen at a higher rate than did the same tissues excised from the warm-adapted animals when measured at the same temperature. The Q_{10} of the orfe tissues was greater than that of the cod tissues, particularly in the 0° C.–10° C. temperature range.

Similar results were reported for goldfish (*Carassius auratus*) brain breis by Freeman (1950). However, for muscle tissue, the oxygen consumption was greater in muscle from warm-adapted than from cold-adapted fish. These findings on muscle were corroborated by the studies of Suhrman (1955).

In an attempt to localize the cellular mechanisms of temperature adaptation, Precht and his co-workers have recently shown alterations in enzyme activity in tissues from animals adapted to various temperatures (Precht, Christophersen and Hensel, 1955). Christophersen and Precht (1952) observed that the catalase activity of the liver of the carp (*Carrasius vulgaris* Nils) adapted to 1° C. was greater than that of liver from fish adapted to 26° C. However, the dehydrogenase activity (rate of decolorization of methylene blue) was found to be greater in the liver of the warm-adapted animals. On the other hand, Precht (1951) found that the liver catalase activity of the eel (*Anguilla vulgaris* L.) adapted to 26° C. was greater than that of liver catalase from animals adapted to 11° C.; the dehydrogenase activity of liver or muscle brei decreased as the adaptation temperature increased; the catalase activity of eel muscle appeared to be independent of the adaptation temperature.

In potato beetles (*Leptinotarsa decemlineata* Say) adapted to 3° C. and 24° C. Precht (1953) found that blood catalase and tissue succinic dehydrogenase were greater in their activity in the cold-adapted than in the warm-adapted insects. The high temperature of adaptation was only 17.5° C.; Precht also observed that higher temperatures (24° C.) greatly increased locomotor activity in these insects with a concomitant increase in dehydrogenase activity.

Materials and Methods

Adaptation of animals. The experimental animals (*Carassius auratus*) were obtained from Grassyfork, Inc., Martinsville, Indiana. These goldfish varied in

weight from 20 to 80 grams. Upon arrival at the laboratory they were put into a series of tanks which were continually being renewed with aerated dechlorinated water. Pulverized Purina Dog Chow was placed into these tanks for one day. After this period the fish were transferred to continuously aerated water in the adaptation tanks, which consisted of seven-gallon aquaria immersed in water at 10° and 30° C. The fish were kept in these adaptation tanks for two weeks without feeding prior to experimentation. The water in the tanks was completely changed every week.

Preparation of tissues for manometric studies. Following the adaptation period a fish was removed from the adaptation tank, blotted with a paper towel, and weighed. The animals were then killed by severing the spinal cord immediately posterior to the head with a pair of scissors. The brain was removed first and blotted to remove blood and meninges; then the body cavity was opened by two connecting incisions—one median ventral and one ventral transverse. Thus, the liver could be removed piece-wise by scraping it away from the intestine, spleen, and gall bladder. Then the gill filaments were cut away from the gill arches, washed in frog Ringer's, and placed in the medium described below. Gills are quite thin and thus may be used in a Warburg vessel without homogenization or mincing.

Ringer's Solution

NaCl	6.5 g./l.	
KCl	0.15 g./l.	
$CaCl_2$	0.12 g./l.	70 parts
$NaHCO_3$	0.20 g./l.	
Na_2HPO_4	0.01 g./l.	

0.3 *M* glucose	5 parts
0.16 *M* Na-pyruvate	4 parts
0.1 *M* Na-succinate	7 parts
0.16 *M* Na-L glutamate	4 parts

Phosphate buffer pH 7.5

13 parts	0.021 *M* Na_2HPO_4
1 part	0.160 *M* KH_2PO_4

The above mixture was kept frozen in the refrigerator prior to use. New mixtures were made up weekly. The medium is similar to that prepared by Krebs (1950) for mammalian tissues. Each vessel contained 2.5 ml. of the above medium and gill filaments plus 0.2 ml. of 10 per cent KOH and fluted Whatman No. 1 filter paper in the center well.

Brain and liver homogenates did not survive well at temperatures above 20° C. Thus, if the oxygen consumption were measured at temperatures of 10°, 18°, and 26° C. successively with the same tissues, it was observed that the oxygen consumption at 26° C. was decidedly lower than if the Q_{O_2} had been measured initially at this temperature. The homogenate technique was discarded, therefore, in favor of the following: The tisues were removed from the animal and placed on filter paper on a Petri dish over ice. The tissues were then minced with a razor blade and placed into the previously mentioned medium. It was found that the oxygen consumption of a given sample of tissue could be measured at two tem-

peratures (14° and 22° C., 10° and 30° C.) without appreciable loss of activity at the higher temperature.

Cyanide inhibition of respiration. The method used in these studies was essentially that of Robbie (1948). The center well contained 0.4 ml. of 0.42 *M* $Ca(CN)_2$ in 10 per cent $Ca(OH)_2$. This mixture equilibrates with the vessel fluid to give a final cyanide concentration of 10^{-3} *M*. However, the equilibration time is about one hour at 26° C. and to alleviate this delay 0.1 ml. of 2.8×10^{-2} *M* KCN was placed into the vessel fluid of 2.7 ml., thus giving a final concentration of 10^{-3} *M* cyanide. Samples of the vessel fluid were removed at the end of an experiment and analyzed for cyanide by the phenolphthalin method of Robbie (1948). It was found that the equilibrium concentration of cyanide was not appreciably altered by the above procedure. The control vessels contained 0.4 ml. of 10 per cent $Ca(OH)_2$.

Iodoacetate inhibition of respiration. Since iodoacetate produces its effect on living systems by combining with SH groups of various enzymes, it is to be expected that its action will be non-specific. However, Adler and co-workers (1938) have shown that triose phosphate dehydrogenase (TDH) is more sensitive to iodoacetate than a number of other sulfhydryl enzymes. Recently Kelly and co-workers (1955) have used iodoacetate in small concentration (5.4×10^{-4} *M*) to block glycolysis in adrenal tissue Thus, it was hoped that a similar study utilizing goldfish gills would yield information concerning the activity of the hexose monophosphate shunt in this tissue.

The following medium was used:

Control		*Experimental*	
0.3 *M* glucose	5 parts	0.3 *M* glucose	5 parts
Phosphate buffer	30 parts	Phosphate buffer	30 parts
Ringer's solution	70 parts	15 mg. iodoacetic acid in 100 ml. Ringer's solution	70 parts

Thus, the final concentration of iodoacetate in the vessel was 5.4×10^{-4} *M*.

It was found that iodoacetate produced only a small inhibitory effect on the oxygen consumption of whole gills. Before considering that this lack of marked inhibition is due to a high level of activity of the hexose monophosphate shunt it was necessary to know whether the iodoacetate was entering the cells. At pH 7.5 the iodoacetic acid is highly ionized, and if one assumes that only the un-ionized form enters the cells, then a method for increasing the amount of the un-ionized form would be to lower the pH. However, a drop of 2–3 pH units would decidedly inhibit oxygen consumption; thus the homogenate technique was employed to facilitate diffusion of the iodoacetic acid into the cells.

The results of the rate-temperature studies for goldfish brain, liver, and gills are given in tabular form in Tables I and II and in graphic form in Figure 1. The data for the cyanide and iodoacetate inhibition studies are given in Table III. Two tests were used for the level of significance: Student's Test as modified by Fisher and reported by Patau (1943) and the Mann-Whitney U Test as reported by Siegel (1956). The latter test is much simpler to run than the *t* test, and since it is a nonparametric test as opposed to the parametric *t* test, one need not concern

TABLE I

Oxygen consumption of brain and liver from goldfish adapted to 10° C. and from goldfish adapted to 30° C.

Temperature of measurement in ° C.	Q_{O_2} (μl./mg. dry weight/hour)				P value	
	Adaptation temperatures					
	10° C.	Number of animals	30° C.	Number of animals	*t*	M-W
Brain						
10	0.813	5	0.700	5	0.37	0.11
14	0.992	5	0.887	5	0.90	0.42
22	1.53	5	1.50	4	0.93	0.45
30	2.27	4	2.18	4	0.92	0.17
Liver						
10	0.621	4	0.454	4	0.76	0.10
14	0.907	6	0.649	5	0.04	0.09
22	1.39	3	0.849	3	0.08	0.05
26	1.36	8	1.19	6	—	0.286
30	1.44	4	1.41	4	—	0.56

himself with the stringent assumptions of the *t* test. The probabilities for assuming random distribution are given for both tests in Tables I–III. The five per cent level is taken as the minimum level of significance.

Brain and liver oxygen consumption. The differences in oxygen consumption for brain and for liver from goldfish adapted to 10° C. and 30° C. as shown in Table I and Figure 1 are small and not statistically significant.

Gill oxygen consumption. The oxygen consumption of gills from goldfish adapted to 10° C. was in all cases greater than that of gills from fish adapted to 30° C. as shown in Table II and Figure 1c. However, in the sequence of meas-

TABLE II

Oxygen consumption of gills from goldfish adapted to 10° C. and from goldfish adapted to 30° C.

Temperature of measurement in ° C.	Month of study	Q_{O_2} (μl./mg. dry weight/hour)				P value	
		Adaptation temperature					
		10° C.	Number of animals	30° C.	Number of animals	*t*	M-W
10	August	0.405	3	0.255	3	0.07	0.002
14	July	0.706	3	0.448	3	0.14	0.10
22	July	1.37	4	0.858	4	0.001	0.014
30	August	1.60	3	1.00	3	0.013	0.05
10	February	0.257	8	0.106	6	0.002	0.002
18	February	0.557	8	0.262	6	0.0006	0.001
26	February	1.074	8	0.522	4	0.0002	0.002

urements from July to February all of the curves (Fig. 1c) are shifted down on the rate axis. This indicates a seasonal variation upon which temperature acclimation is superimposed. Homogenized gills (controls for the IAA experiments) had a lower oxygen consumption, 0.553 μl/mg./hr. for the 10° C. gills and 0.193 μl/mg./hr. for the 30° C. gills than did whole gills (Table II), but the Q_{0_2}

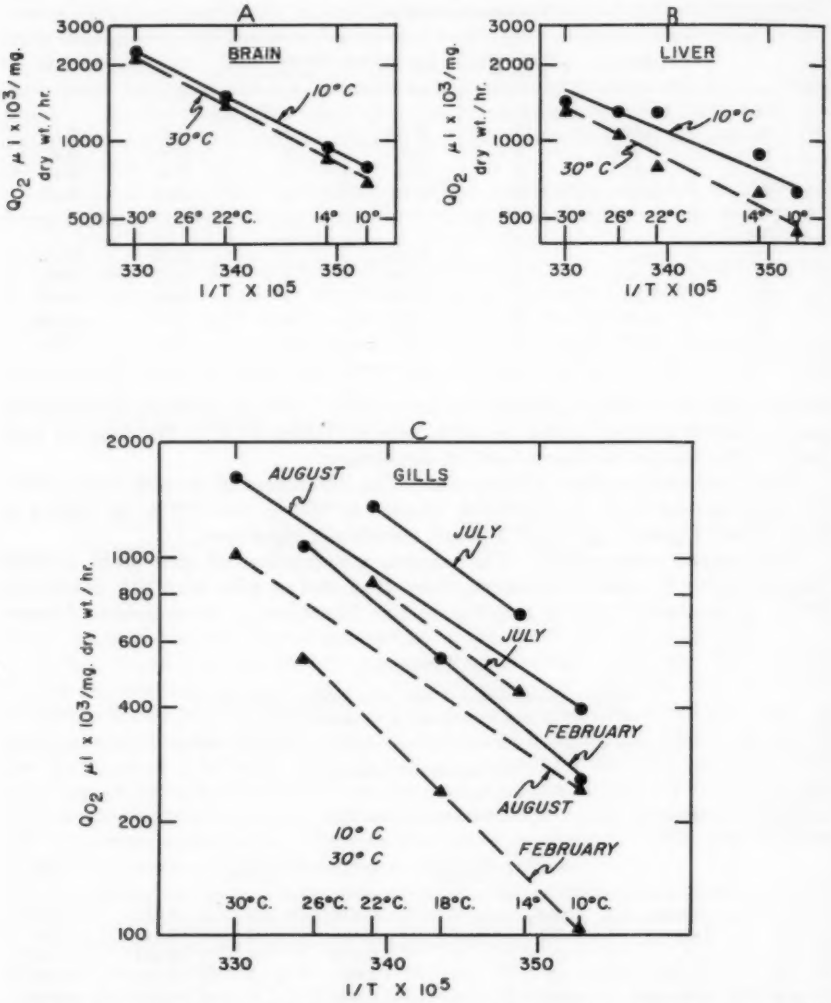

FIGURE 1. Arrhenius plot of the oxygen consumption of brain (a), liver (b), and gills (c) from goldfish adapted to 10° C. (solid lines) and from goldfish adapted to 30° C. (broken lines). See Tables I and II for statistical data.

for gill homogenates from cold-adapted fish was significantly higher than homogenates from warm-adapted fish.

Cyanide inhibition of oxygen consumption. The results of the effects of 10^{-3} *M* cyanide on the oxygen consumption of goldfish liver and gills are summarized in Table III. A small non-significant difference is noted for liver, while the gills from fish adapted to 30° C. were more resistant to cyanide poisoning than gills from fish adapted to 10° C.

Iodoacetate inhibition of oxygen consumption. The data for this group of experiments are summarized in Table II. Iodoacetate had little or no effect on the oxygen consumption of intact gills, yet IAA strongly inhibited the gill homogenates. This suggests that permeability is the limiting factor. This is in contrast to the cyanide studies in which the effect of cyanide was fairly rapid (inhibition was maximum after a 10-minute equilibration period). Since no definite state-

TABLE III

The effect of 10^{-3} M cyanide (Nov.–Dec.–Jan.) and 5.4×10^{-4} M iodoacetic acid (Feb.–March) on the oxygen consumption of tissues from goldfish adapted to 10° C. and from goldfish adapted to 30° C. when measured at 26° C.

Tissues	Per cent inhibition of oxygen consumption				P value	
	Adaptation temperature					
	10° C.	Number of animals	30° C.	Number of animals	*t*	M-W
Cyanide						
Liver	85.9	7	81.2	7	0.17	0.13
Gills (whole)	79.1	7	57.5	7	0.024	0.009
Iodoacetate						
Gills (homogenized)	52.6	7	77.4	7	—	0.027

ments can be made concerning the number of cells broken in the homogenate or of the amount of iodoacetate that actually entered the cells, it is impossible to estimate accurately the amount of metabolic activity accounted for by the hexose monophosphate shunt. However, these results do indicate that gill homogenates from 10° C.-adapted fish are more resistant to iodoacetate poisoning than are gills from fish adapted to 30° C.; this is opposite to the effect of cyanide.

DISCUSSION

In view of the negligible temperature adaptation differences in oxygen consumption observed in goldfish liver and brain, it appears that further studies of oxygen consumption in these tissues would be of doubtful value. On the other hand goldfish gills appear to be an ideal tissue for this type of study in that gills from fish adapted to 10° C. do show a significantly higher rate of oxygen consumption than do gills from fish adapted to 30° C. when measured at temperatures between 10° C. and 30° C.

Figure 2 indicates that there is a definite decrease in oxygen consumption by goldfish gills during the period from July to February regardless of the adaptation temperature. Seasonal variations in oxygen consumption of intact goldfish have not been reported. However, Hoar (1955, 1956) has demonstrated a greater resistance to high temperature in summer than in winter for goldfish adapted to the same temperature.

A comparison of the rate-temperature curves reveals no apparent difference between slopes of oxygen consumption by gills from animals adapted to 10° C. or 30° C. for any given month of measurement. Yet, this does not exclude the possibility that an enzyme or a group of enzymes may change in such a way that the net effect is unnoticeable.

Precht *et al.* (1955), as previously mentioned, have shown that enzymes such as dehydrogenases and catalase change their activity with a change in adaptation temperature. However, he has worked with homogenates and thus may not conclude that a certain enzyme has increased or decreased in concentration, but only that its *activity* has increased or decreased. One must consider the following possibilities as affecting the activity of a certain enzyme:

(1) change in permeability of the cell membrane to substrate molecules;
(2) release within the cell of some activating agent or of some inhibitor;
(3) inactivation of a chemical inhibitor or activating agent initially present within the cell;
(4) increase or decrease of the actual amount of enzyme present;
(5) change in intrinsic properties of the enzyme itself.

Present data do not allow a distinction to be made among these possibilities. However, Kaplan (1954, 1955), Fraser and Kaplan (1955), and Kaplan and Paik (1956) have attempted to describe the Euler effect (an increase in enzyme activity when the enzyme is extracted from the cell) on yeast catalase by progressive elimination of (1) to (4) above, thus leaving (5) as the operating mechanism.

Cyanide inhibition of gill oxygen consumption. The increased resistance of gills from 30° C.-adapted goldfish to 10^{-3} *M* cyanide, as measured by oxygen consumption, may be interpreted in a number of ways.

(1) There is an increase in the amount of one or more of the cytochromes in the gills from 30° C.-adapted fish.
(2) The cytochrome system of the gills from 30° C.-adapted fish is changed in such a manner that it is less sensitive to cyanide.
(3) An oxidative pathway which is less sensitive to cyanide than is the cytochrome system is increased in activity in the gills from 30° C.-adapted fish.

In view of the fact that the oxygen consumption of the gills from 30° C.-adapted fish is lower than that of gills from 10° C.-adapted fish, it does not appear that (1) offers an adequate explanation of the observed differences in cyanide sensitivity. The second possibility (2) must be considered as a possible explanation and merits further study. It seems more probable, however, that in the gills of fish adapted to high temperatures the oxidative pathway is shifted away from the cytochrome system (3). If the activity of the oxidases and aerobic dehydrogenases were increased, this in turn could increase the activity of the peroxidases and catalase.

Iodoacetate inhibition of gill oxygen consumption. If one may assume that iodoacetic acid is primarily blocking triose phosphate dehydrogenase, then it would

appear that the hexose monophosphate shunt is operating at a higher capacity in the gills of 10° C.-adapted fish. Such an increase in shunt activity suggests (Glock and McClean, 1954) a correlation between protein synthesis and the shunt. In cold-adapted fish gills there may exist a higher rate of protein synthesis than in warm-adapted fish gills. This in turn would increase the need of cold-adapted fish gills for oxygen.

These inhibitor studies suggest that the cytochrome system has become less sensitive to cyanide poisoning or that an alternate pathway less sensitive to cyanide than the cytochrome system has increased in activity in gills from fish adapted to 30° C., and that the hexose monophosphate shunt may be more active in gills from 10° C.-adapted fish.

The writer is deeply indebted to Professor C. Ladd Prosser for his helpful suggestions during the experimentation period and during the preparation of the manuscript.

Summary and Conclusions

1. No statistical differences were observed in the oxygen consumption by either brain or liver homogenates from goldfish adapted to 10° C. and from fish adapted to 30° C., when measurements were at temperatures between 10° and 30° C.

2. The oxygen consumption of gills from goldfish adapted to 10° C. was found to be significantly higher at all temperatures of measurement, than the oxygen consumption of gills from goldfish adapted to 30° C.

3. No difference was noted in the slopes of the rate-temperature curves of the gills from fish adapted to 10° and from fish adapted to 30° C. as measured by oxygen consumption.

4. A seasonal variation in the oxygen consumption of goldfish gills was noted, in that the oxygen consumption of the gills decreased from July to February. This was true regardless of the temperature of adaptation. However, significant differences were observed between 10° C. and 30° C. gills throughout the experimental period.

5. The oxygen consumption of gills from fish adapted to 30° C. was inhibited to a lesser extent by 10^{-3} *M* cyanide than was the oxygen consumption of gills from fish adapted to 10° C.

6. The oxygen consumption of gills from goldfish adapted to 10° C. was inhibited to a lesser extent by 5.4×10^{-4} *M* iodoacetate than was the oxygen consumption of gills from fish adapted to 30° C.

7. It is suggested that temperature acclimation may be associated with changes in relative activities of CN^- and IAA sensitive enzymatic pathways.

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STABILITY OF THE CHROMATOPHOROTROPINS OF THE DWARF CRAYFISH, CAMBARELLUS SHUFELDTI, AND THEIR EFFECTS ON ANOTHER CRAYFISH [1]

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The chromatophore systems of three crayfishes have been investigated to a considerable degree. Of these most is known about color changes in the dwarf crayfish, *Cambarellus shufeldti.*

Brown and Meglitsch (1940) found that the sinus gland in the eyestalk of the crayfish *Orconectes immunis* contained a chromatophorotropin that concentrated red pigment. McVay (1942) demonstrated that the central nervous system of this crayfish produced a material that concentrated red pigment.

The chromatophore system of the dwarf crayfish has been the subject of previous investigations by Fingerman (1957a, 1957b) and Fingerman and Lowe (1957a, 1957b). The red pigment dispersed maximally when dwarf crayfish were put on a black background and concentrated maximally when the crayfish were put on a white background. In the supraesophageal ganglia, circumesophageal connectives, and tissues of the eyestalks were chromatophorotropins that dispersed as well as concentrated red pigment (Fingerman, 1957a).

Behavior of red pigment on isolated portions of the carapace of *Cambarellus* that had been on black and on white backgrounds was also investigated (Fingerman, 1957b). Red pigment had an inherent tendency to concentrate nearly maximally when the chromatophores were no longer controlled by hormones. Reciprocal blood transfusions between specimens of *Cambarellus* that had been on black and on white backgrounds for two hours revealed that the blood always contained pigment-dispersing and -concentrating hormones. The degree of pigment dispersion at any time appeared to be due to the relative quantity of each antagonist in the blood.

Fingerman and Lowe (1957a) determined the effects of maintenance of specimens of *Cambarellus* on black and on white backgrounds for periods up to three weeks. The rates of red pigment dispersion and concentration in intact crayfish decreased progressively. In addition, red pigment gradually lost its inherent ability to concentrate after isolation.

The titers of chromatophorotropins in the circumesophageal connectives of dwarf crayfish that had been on a black or a white background for two weeks changed significantly relative to the titers in crayfish that had been on the same shade of background for two hours (Fingerman and Lowe, 1957a). They found that the hormone not needed for proper adaptation to one of the backgrounds, *e.g.*, red pigment-dispersing hormone of crayfish on a white background, was stored in the circumesophageal connectives. The quantities of chromatophorotropins in the blood also

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changed. The hormone that was stored in the circumesophageal connectives of crayfish kept on a black or a white background for two weeks decreased in the blood and the hormone needed in the blood for background adaptation increased. These data provided evidence that the secretory products of the central nervous system were physiologically involved in the color change process.

Fingerman and Lowe (1957b) suggested that the chromatophorotropins in the eyestalks of dwarf crayfish were different from those in the circumesophageal connectives. Evidence was based on differences in rates of disappearance of the hormones in extracts of the eyestalks and circumesophageal connectives maintained at room temperature.

Chromatophorotropins that dispersed and concentrated red pigment were found in the eyestalks, supraesophageal ganglia, and circumesophageal connectives of the crayfish *Orconectes clypeatus* by Fingerman (1958). When specimens of *Orconectes clypeatus* were kept on a black background their red pigment dispersed maximally but when the crayfish were transferred to a white pan this pigment concentrated to an intermediate state only. Maximal red pigment concentration did not occur in specimens kept under constant illumination on a white background for 32 days. Hormones that dispersed red pigment could be demonstrated only by direct application of extracts to chromatophores on isolated portions of carapace. Direct injection of extracts did not produce red pigment dispersion.

The current concept of the origin of chromatophorotropins is that they are all neurosecretory products. Those found in the sinus gland originate in the medulla terminalis X organ as granules which migrate through axons to the sinus gland where they are stored (Bliss, Durand and Welsh, 1954). Fingerman and Aoto (unpublished observations) have found cytological evidence of neurosecretion in the supraesophageal ganglia, circumesophageal connectives, and optic ganglia of dwarf crayfish.

The present investigation was undertaken to shed light on several questions that have arisen as a result of the studies summarized above. For example, (1) since direct injection of tissue extracts of *Orconectes* into specimens of *Orconectes* did not cause pigment dispersion, we wished to learn whether tissue extracts from *Cambarellus* could disperse red pigment in specimens of *Orconectes* and vice versa. (2) The possibility that the chromatophorotropins found in the eyestalk are different from those found in the supraesophageal ganglia and circumesophageal connectives was also investigated further.

Materials and Methods

Adult specimens of the dwarf crayfish, *Cambarellus shufeldti*, and immature specimens of the crayfish *Orconectes clypeatus* were collected at Hickory, Louisiana, for use in the experiments described below. The specimens of both species used in the experiments were about 20 mm. long. The crayfishes were kept in aquaria that contained tap water approximately one inch deep. Air-conditioning kept the temperature of the laboratory between 25 and 27° C. The dark red chromatophores in the portion of the carapace dorsal to the heart were staged according to the system of Hogben and Slome (1931) by the senior author. Stage 1 represented maximal concentration of pigment, stage 5 maximal dispersion, and stages 2, 3, and 4 the intermediate conditions.

Tissue extracts were routinely prepared as follows. The organs to be assayed were dissected out and placed in Van Harreveld's solution (Van Harreveld, 1936). When the desired number of each organ was available the organs were transferred with a minimum of saline to a glass mortar, triturated, and suspended in a sufficient volume of Van Harreveld's solution to yield the desired concentration.

Crayfish that received injections of extracts had one eyestalk removed at least 12 hours prior to the experiment. Brown, Webb and Sandeen (1952) and Fingerman (1957a) found that the responses of one-eyed individuals to chromatophorotropins were greater than the responses of intact specimens, presumably because the presence of both eyestalks made the organisms more capable of antagonizing injected hormones.

Brown and Meglitsch (1940) and McVay (1942) were forced to use large specimens of *Orconectes immunis* whose exoskeleton was opaque. These investigators, therefore, applied extracts directly to the chromatophores on portions of the carapace removed from the body. The specimens used herein were small enough so that their carapaces were sufficiently transparent to allow accurate, direct observation of the underlying chromatophores.

For convenience and to save space the hormone that concentrated red pigment will be referred to as RPCH (red pigment-concentrating hormone) and that which dispersed red pigment as RPDH (red pigment-dispersing hormone). Use of the same letters for hormones in the eyestalks and in the supraesophageal ganglia plus the circumesophageal connectives does not imply that the chromatophorotropins have the same molecular structure.

Experiments and Results

Analysis of variation to be expected when the same extract is injected into two groups of dwarf crayfish

The aim of this experiment was to determine how much of the variance between two sets of data in the results presented below would be due to differential response of the crayfish used for assay and how much to differences in titers of chromatophorotropins in the extracts. To solve this problem an extract of the supraesophageal ganglia, with the circumesophageal connectives attached, of *Cambarellus* was prepared with a final concentration of one-third of a complement per 0.02 ml. Van Harreveld's solution. Ten one-eyed dwarf crayfish were then placed into each of two white enameled pans containing tap water; five were then placed into a third white pan. In like manner 25 were distributed among three black enameled pans. The crayfish in the white pans had been on a white background for at least one hour and were inspected prior to the experiment to be certain that their red pigment was maximally concentrated (stage 1). Crayfish in black pans had maximally dispersed red pigment (stage 5).

The crayfish in the two pans with five crayfish were each injected with 0.02 ml. Van Harreveld's solution as a control. The crayfish in the remaining pans were then injected with the extracts. Each crayfish in one white and one black pan was injected from the same syringe with 0.02 ml. of extract. The average stage of the red chromatophores of the crayfish in each pan was determined 15, 30, 60, 90 and 120 minutes from the time of injection.

The results are presented in Figure 1. As evident from inspection of the figure, RPCH and RPDH were present. The maximal effect of the former preceded that of the latter, the typical situation with extracts of supraesophageal ganglia and circumesophageal connectives (Fingerman, 1957a). The results of the two groups that received extracts were extremely similar, if not identical, since the difference between two consecutive determinations of the average chromatophore stage of the same group of crayfish may be 0.2 of a unit, but never more. The sum of the differences between the total of the average chromatophore indices for corresponding

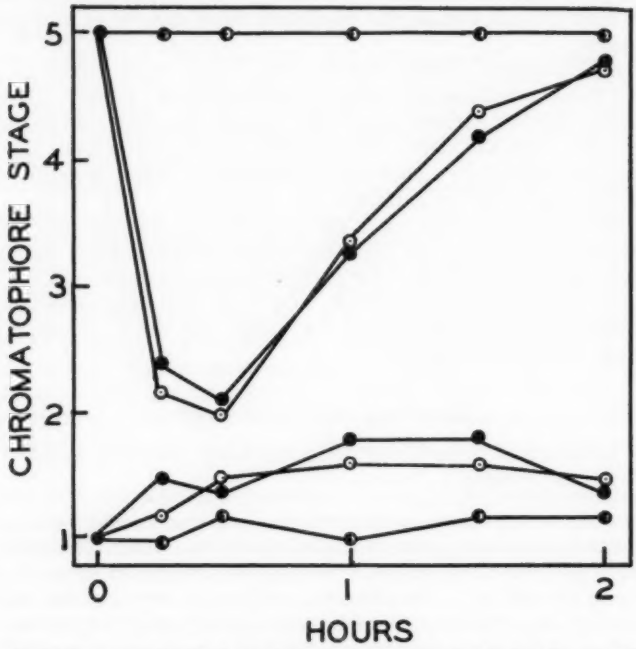

FIGURE 1. Responses of red chromatophores of one-eyed *Cambarellus* on black and on white backgrounds to the same extract of supraesophageal ganglia, with the circumesophageal connectives attached. Dots and circles, two groups that received the same extract; half-filled circles, control.

groups of crayfish was 0.6 unit for dispersing activity and 0.1 unit for concentrating activity. Results appreciably different from these values may, therefore, be considered as due to a significant difference in the quantities of chromatophorotropins in the extracts themselves and not to the crayfish used for the assay.

Responses of dwarf crayfish to chromatophorotropins of Cambarellus and Orconectes

The aim of this experiment was to compare the effects of extracts of eyestalks and supraesophageal ganglia, with the circumesophageal connectives attached, of dwarf crayfish and *Orconectes* upon the dark red chromatophores of dwarf crayfish.

Since direct injection of freshly prepared extracts of eyestalks and supraesophageal ganglia plus the circumesophageal connectives of *Orconectes* into specimens of *Orconectes* never produced red pigment dispersion, we wished to learn whether these extracts could disperse red pigment in specimens of *Cambarellus*.

Both eyestalks and the supraesophageal ganglia, with the circumesophageal connectives attached, of dwarf crayfish and *Orconectes* were removed and extracted as described above, so that the final concentration was one-third of a complement per 0.02 ml. Five one-eyed dwarf crayfish with maximally concentrated red pigment were placed into each of five white pans that contained aerated tap water. In like manner five one-eyed crayfish with maximally dispersed red pigment were placed into each of five black pans. The crayfish were injected and the chromatophores staged in the manner described in the first experiment.

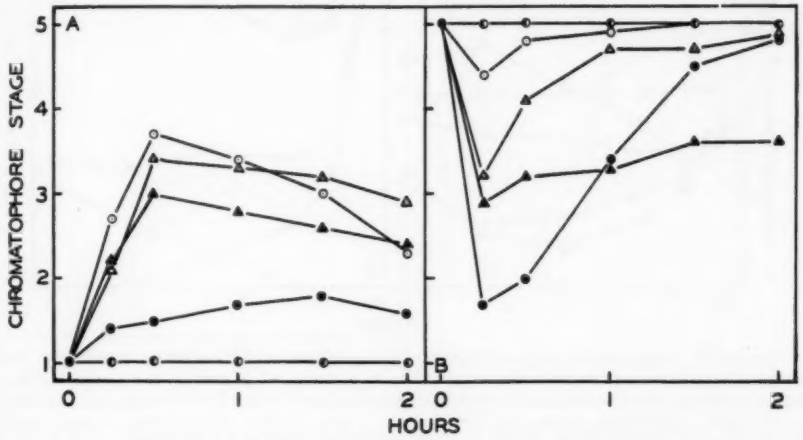

FIGURE 2. Responses of the red chromatophores of one-eyed *Cambarellus* on a white background (A) and on a black background (B) to extracts of eyestalks and supraesophageal ganglia, with the circumesophageal connectives attached, of *Cambarellus shufeldti* and *Orconectes clypeatus*. Half-filled circles are the controls. Open symbols are eyestalks, closed symbols nervous tissue. Circles, tissue of *Cambarellus;* triangles, tissue of *Orconectes*.

The results are presented in Figure 2 where each point represents the average of 10 crayfish. As evident from the figure, each extract contained RPCH and RPDH. The eyestalks of dwarf crayfish and *Orconectes* contained more RPDH and less RPCH than the supraesophageal ganglia plus the circumesophageal connectives. Significantly, the eyestalks and supraesophageal ganglia plus the circumesophageal connectives of *Orconectes* contained sufficient RPDH to produce an appreciable response in dwarf crayfish but no red pigment dispersion occurred when extracts of these tissues were injected into specimens of *Orconectes* (Fingerman, 1958).

Responses of Orconectes to chromatophorotropins of dwarf crayfish and Orconectes

Since direct injection of extracts of eyestalks and supraesophageal ganglia plus the circumesophageal connectives of *Orconectes* into dwarf crayfish did produce red

pigment dispersion, we wished to determine the results of the reciprocal experiment. Red pigment of the specimens of *Orconectes* used in the assay was in an intermediate state of dispersion since red pigment in this species does not concentrate maximally when specimens are placed on a white background (Fingerman, 1958).

The results are presented in Figure 3 where each point represents the average of 10 crayfish. Extracts of tissues from *Orconectes* did not produce red pigment dispersion; RPCH alone was evident. As had been found previously (Fingerman, 1958), supraesophageal ganglia and circumesophageal connectives were more potent sources of RPCH than eyestalks.

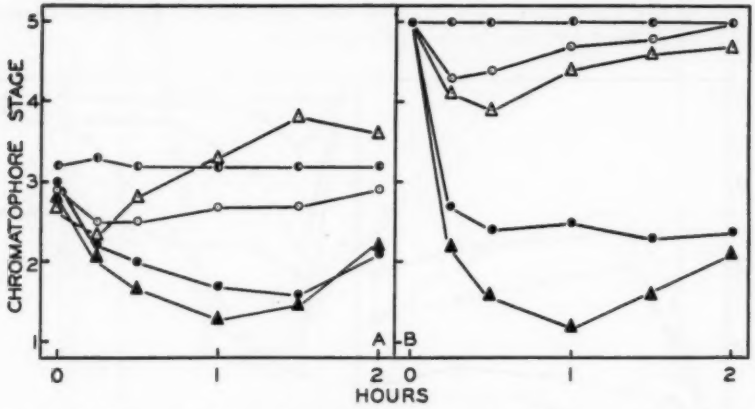

FIGURE 3. Responses of the red chromatophores of one-eyed *Orconectes* on a white background (A) and on a black background (B) to extracts of eyestalks and supraesophageal ganglia, with the circumesophageal connectives attached, of *Orconectes clypeatus* and *Cambarellus shufeldti*. Half-filled circles are the control. Open symbols are eyestalk, closed symbols nervous tissue. Circles, tissue of *Orconectes;* triangles, tissue of *Cambarellus*.

The responses of specimens of *Orconectes* on black and on white backgrounds to extracts of supraesophageal ganglia, with the circumesophageal connectives attached, of dwarf crayfish and *Orconectes* were qualitatively similar. Concentration but no dispersion of red pigment in *Orconectes* was evident in spite of the fact that RPCH and RPDH were present in these extracts (Fig. 2).

Eyestalk extracts of *Cambarellus*, however, caused a transitory concentration of red pigment in *Orconectes* that was followed by dispersion. Concentration must have been caused by the small amount of RPCH in the eyestalks of dwarf crayfish and dispersion by the large amount of RPDH shown in Figure 2. Extracts of eyestalks of both species produced equal red pigment dispersion in dwarf crayfish but the eyestalk extract of *Orconectes* contained much more RPCH (Fig. 2). This difference was probably a contributing factor to the lack of red pigment dispersion in *Orconectes* with extracts of tissues from *Orconectes*, because the large amount of RPCH would hinder the expression of RPDH whereas in the eyestalk of *Cambarellus* the titer of RPDH so overbalanced the titer of RPCH that dispersion could not occur. The failure of extracts of *Orconectes* to disperse red pigment in *Orconectes*

suggests that the titers of chromatophorotropins in the blood at the time of injection are important or else when the extracts are injected materials are secreted to antagonize the added hormones.

Influence of time and temperature upon chromatophorotropins of dwarf crayfish

This experiment was designed to determine the effects of boiling and maintenance at room temperature upon the titers of RPCH and RPDH in the supraesophageal ganglia with the circumesophageal connectives attached of dwarf crayfish. Twenty organs were removed and extracted in 1.2 ml. Van Harreveld's solution. The extract was then divided into two equal portions. One fraction was placed in

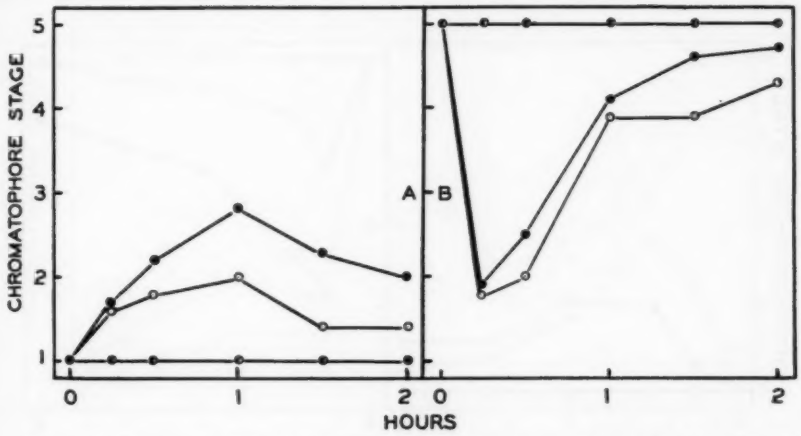

FIGURE 4. Responses of the red chromatophores of one-eyed *Cambarellus* on white (A) and on black (B) backgrounds to boiled (dots) and unboiled (circles) portions of the same extract of supraesophageal ganglia, with the circumesophageal connectives attached, of *Cambarellus* injected immediately after preparation. Half-filled circles, control.

boiling water for 30 seconds and cooled to room temperature. The boiled and unboiled extracts were then assayed on specimens in black and in white pans. The extracts were kept in the syringes on a table top at room temperature for 120 minutes and then assayed again.

The results are presented in Figures 4 (zero time injection) and 5 (120 minute injection). Boiling appeared to activate an inactive form of RPDH but had little, if any, effect on RPCH. Even more striking differences were apparent between the responses of the crayfish to boiled and unboiled extracts kept for 120 minutes at room temperature. The responses of the crayfish injected with the unboiled aged extract were the same as those observed by Fingerman and Lowe (1957b). The amount of RPCH had decreased considerably and the amount of RPDH had increased. In contrast, RPDH in the boiled preparation disappeared at a faster rate than RPCH. The latter showed no decreased potency. If anything, a slight increase was evident, probably due to the degeneration of its antagonist.

These results can be explained by assuming that RPDH exists in the neurosecretory cells in a functional form and a non-functional one which may be activated either by boiling or being kept at room temperature for two hours. Activation probably represents release of bound hormone from the interior of neurosecretory granules as has been shown with catechol amines by Hillarp and Nilson (1954) and Blaschko, Hagen and Welch (1955). Decrease in the quantity of the active form because of its instability occurs simultaneously with release of additional hormone from the neurosecretory granules in unboiled extracts. Inactivation of RPDH would occur in boiled extracts but no further release from the granules because boiling caused the immediate release of all bound RPDH from the neurosecretory granules. To illustrate, at the start of the experiment boiled extract caused more red pigment dispersion than unboiled (Fig. 4A). During the two hours at room

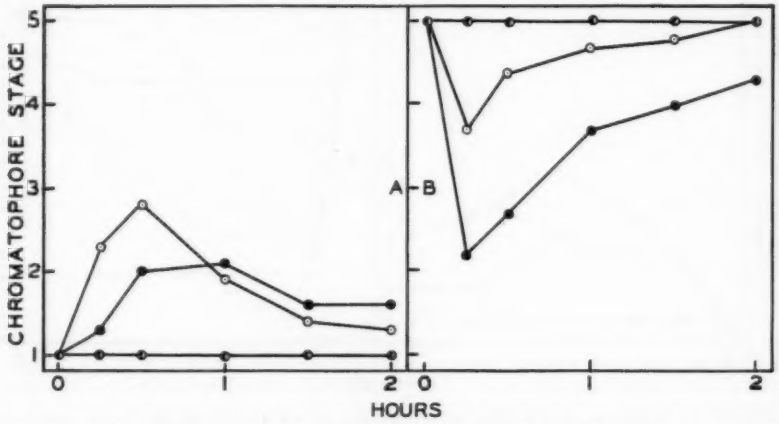

FIGURE 5. Responses of the red chromatophores of dwarf crayfish on white (A) and on black (B) backgrounds to the same extracts of Figure 4 but injected after 120 minutes at room temperature. See Figure 4 for key to symbols.

temperature RPDH was becoming inactivated. Since in the boiled extract no inactive hormone would be available, degeneration alone occurred so that the net effect was decreased potency (Fig. 5A). In the case of the unboiled extract, during the two hours at room temperature the active form was being released from the neurosecretory granules at a faster rate than the functional hormone was becoming inactivated so that the net effect was an increased titer of RPDH. The same logic apparently does not apply to RPCH. Boiling seemed to stabilize the molecule so that it was not inactivated as was RPCH of unboiled extracts.

Stability of chromatophorotropins in dried tissue

The object of this experiment was to determine the effects of drying for two hours on RPCH and RPDH to rule out the possibility that the results of the previous experiment were due to the use of triturated tissues and not to the nature of

the neurosecretory granules or the hormones. Supraesophageal ganglia, with the circumesophageal connectives attached, of dwarf crayfish were dissected out and cut in half longitudinally. One batch was triturated immediately and re-suspended in sufficient Van Harreveld's solution to yield a final concentration of one-third of a complement per 0.02 ml. The second set was dispersed in the bottom of a glass mortar and allowed to dry at room temperature for 120 minutes, then triturated and suspended in sufficient Van Harreveld's solution for a final concentration of one-third of a complement per 0.02 ml. The freshly prepared extract was assayed and two hours later the extract of the dried tissues was assayed.

The results are presented in Figure 6 where each point represents the average of 10 crayfish. The results of drying the tissue for two hours were qualitatively the same as those observed with extracts left at room temperature for two hours. The

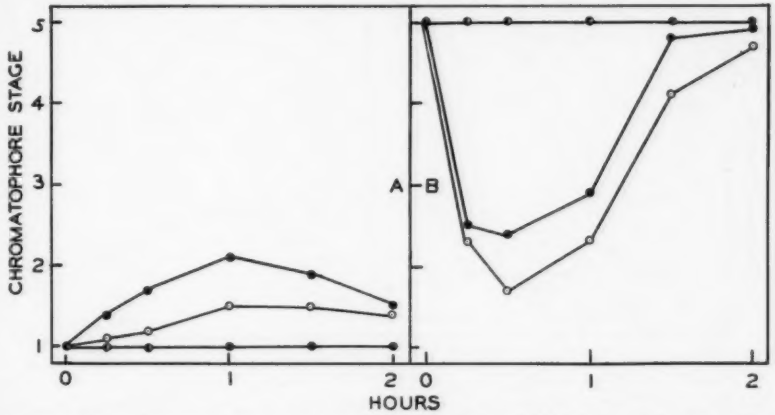

FIGURE 6. Responses of the red chromatophores of one-eyed dwarf crayfish on white (A) and on black (B) backgrounds to extracts of supraesophageal ganglia, with the circumesophageal connectives attached of *Cambarellus*. Circles, extracted at time of removal; dots, dried at room temperature for 120 minutes; half-filled circles, control.

extracts of dried tissues contained more RPDH and less RPCH than the extracts of fresh tissue. The same result was obtained when the extracts were left at room temperature for two hours. Therefore, the changes depicted in Figures 4 and 5 were not due solely to the aqueous environment of the extract but were mainly due to the instability of the hormones and probably of the neurosecretory granules in which the hormones are present when first formed.

Fractionation of chromatophorotropins in the sinus glands and supraesophageal ganglia plus the circumesophageal connectives of dwarf crayfish

The object of this experiment was to determine the relative solubilities of RPDH and RPCH from the sinus gland and the supraesophageal ganglia plus the circumesophageal connectives in absolute ethyl alcohol. In this way further information concerning the similarity of the corresponding molecules from the two sources would be obtained since Fingerman and Lowe (1957b) had obtained preliminary

evidence that RPDH and RPCH in the eyestalks were different from the materials in the circumesophageal connectives of dwarf crayfish with the same functions.

The method employed was described in detail by Fingerman (1956). Six supraesophageal ganglia, with the circumesophageal connectives attached, were placed on a glass slide. The excess moisture was removed and the tissue was then smeared with a glass pestle. In like manner, six sinus glands were removed with a minimum of other tissues from eyestalks and smeared on a glass slide. The hormones in the smeared tissues were then extracted by dropping one ml. 100% ethyl alcohol from a syringe onto the tilted slide and collecting the alcohol in a watch glass. The alcohol was allowed to evaporate and the residue was re-suspended in 0.24 ml. Van Harreveld's solution.

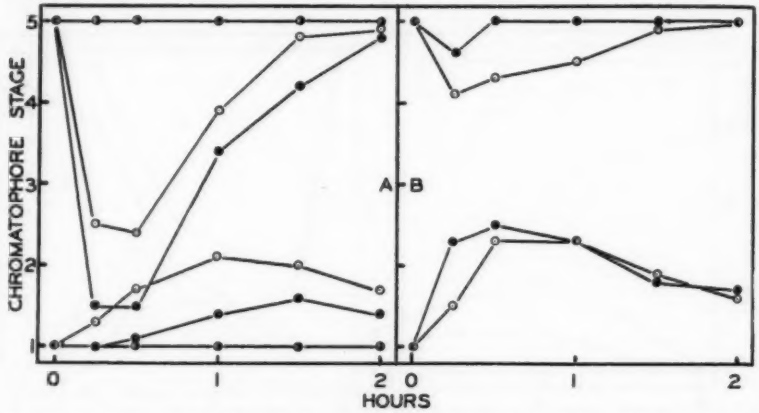

FIGURE 7. Responses of the red chromatophores of dwarf crayfish on black and on white backgrounds to extracts of supraesophageal ganglia, with the circumesophageal connectives attached (A), and sinus glands (B). Dots, alcohol-soluble fraction; circles, alcohol-insoluble fraction; half-filled circles, control.

After the few remaining drops of alcohol had evaporated from the glass slide the tissues were again extracted by placing 0.24 ml. Van Harreveld's solution on the tissues for two to three minutes. The fluid was then taken up in a syringe. The four fractions were then assayed.

The results are presented in Figure 7 where each point represents the average of 10 crayfish. As evident from the left half of the figure (nervous tissue), the alcohol-soluble fraction contained more RPCH and less RPDH than the alcohol-insoluble fraction. Consideration of the results obtained with the sinus glands revealed the reverse situation prevailed. The conclusion may, therefore, be drawn that the chromatophorotropins of the supraesophageal ganglia plus the circumesophageal connectives are different from those of the sinus gland.

Enzymatic inactivation of chromatophorotropins of Cambarellus

This set of experiments was designed to shed further light on what happens to chromatophorotropins after they have been secreted into the blood. Carstam

(1951) showed that the hypodermis of the prawn *Leander adspersus* contains an enzyme capable of inactivating chromatophorotropins from the sinus gland. Fingerman and Lowe (1957b) showed that the presence of hypodermis of dwarf crayfish likewise hastened the inactivation of chromatophorotropins in nervous tissue. We decided, therefore, to investigate further the nature of this inactivation.

For the first experiment of the series supraesophageal ganglia, with the circumesophageal connectives attached, were removed from dwarf crayfish and triturated with a sufficient volume of Van Harreveld's solution to yield 0.90 ml. extract with a concentration of one-third complement per 0.02 ml. Two-hundredths ml. of this extract was then injected into each of five one-eyed crayfish on a white background and into a like number on a black background. The entire carapace had, in the

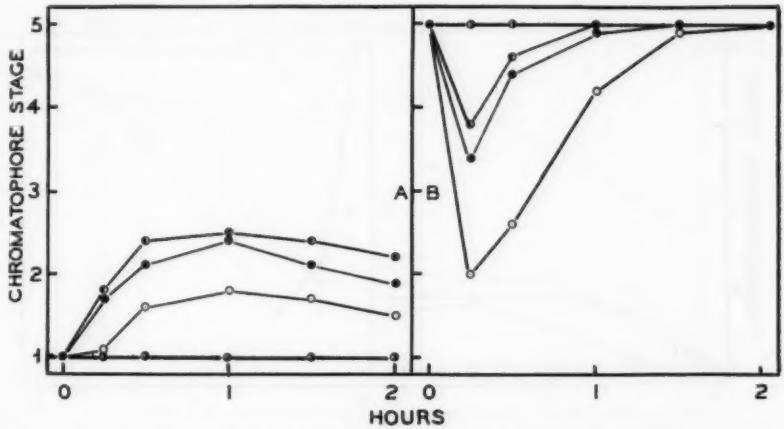

FIGURE 8. Responses of the red chromatophores of *Cambarellus* on white (A) and on black (B) backgrounds to unboiled extracts of supraesophageal ganglia, with the circumesophageal connectives attached, of dwarf crayfish. Circles, extract administered immediately after boiling; dots, after two hours exposure to boiled carapace; circles filled on left side, after two hours exposure to unboiled carapace; circles filled on right side, control.

meantime, been removed from six dwarf crayfish. Three of the carapaces were boiled to destroy the proteins in the hypodermis. The six carapaces were then placed on individual glass depression slides. Into each depression containing a carapace was placed 0.1 ml. of the extract. The slides were then covered to minimize evaporation. After the extract had been exposed to the carapaces for two hours, the fractions that had been exposed to boiled and unboiled carapace were collected separately and assayed. The results are presented in Figure 8 where each point represents the average of 10 crayfish.

The unboiled extract exposed to the boiled and unboiled pieces of carapace behaved just as the unboiled extract shown in Figures 4 and 5. After two hours of exposure to both boiled and unboiled carapace the unboiled extract showed an increase in red pigment-dispersing potency and a decrease in RPCH. The same results were obtained when the unboiled extract was simply left at room temperature

for two hours. The extract that had been exposed to unboiled carapace, however, contained more RPDH and less RPCH than the extract that had been in contact with boiled carapace. Presumably, this difference was due to preferential inactivation of RPCH by an enzyme in the hypodermis of the unboiled carapace. This enzyme must have been destroyed in the boiled carapaces. Apparently, the enzyme destroyed RPCH in preference to RPDH and for this reason the latter was more abundant after two hours of exposure. Spontaneous release of active RPDH from the neurosecretory granules also must have occurred during the two hours of exposure to the carapace.

Since boiled extract kept for two hours had less RPDH than was present at the time of heating (Figs. 4 and 5), the logical sequel to the experiment just described wherein inactivation of the hormones was not great, was to expose boiled extracts to

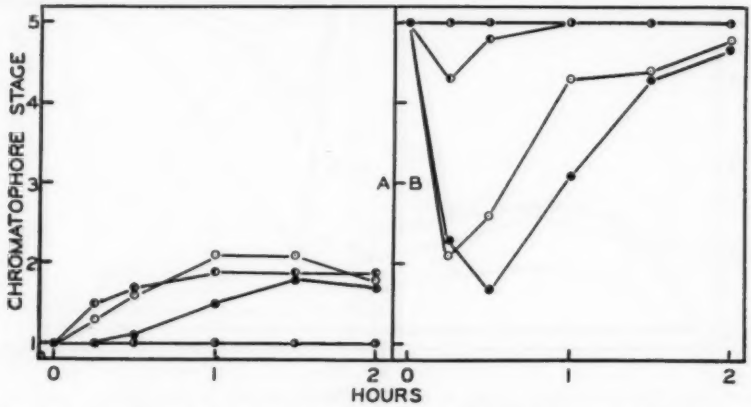

FIGURE 9. Responses of the red chromatophores of *Cambarellus* on white (A) and on black (B) backgrounds to boiled extracts of supraesophageal ganglia, with the circumesophageal connectives attached, of dwarf crayfish. Circles, extract administered immediately after boiling; dots, after two hours exposure to boiled carapace; circles filled on left side, after two hours exposure to unboiled carapace; circles filled on right side, control.

boiled and unboiled carapaces for two hours. Furthermore, as mentioned above, the titer of RPCH in boiled extracts showed no tendency to decrease for at least two hours at room temperature so that any change in the titer of this hormone would have been enzymatically induced.

Extracts for this experiment were prepared and handled in the same manner as those for the first experiment of this series with the one exception that the extract was immersed in boiling water for 30 seconds, cooled, and then placed on the depression slides. The results of the second experiment are shown in Figure 9. Each point represents the average of 10 crayfish.

The extract exposed to boiled carapace for two hours behaved exactly like the boiled extract of Figure 5; the titer of RPDH decreased markedly whereas no tendency for RPCH to diminish was apparent. However, the results with the boiled extract exposed to unboiled carapace were quite different from those obtained with

extracts exposed to unboiled carapace. The quantity of RPCH decreased markedly, presumably due to enzymatic inactivation. The quantity of RPDH showed little or no decrease, probably because so much of its antagonist was destroyed that a lower titer of RPDH was able to exert a sizeable effect. Here also a preferential inactivation of RPCH occurred.

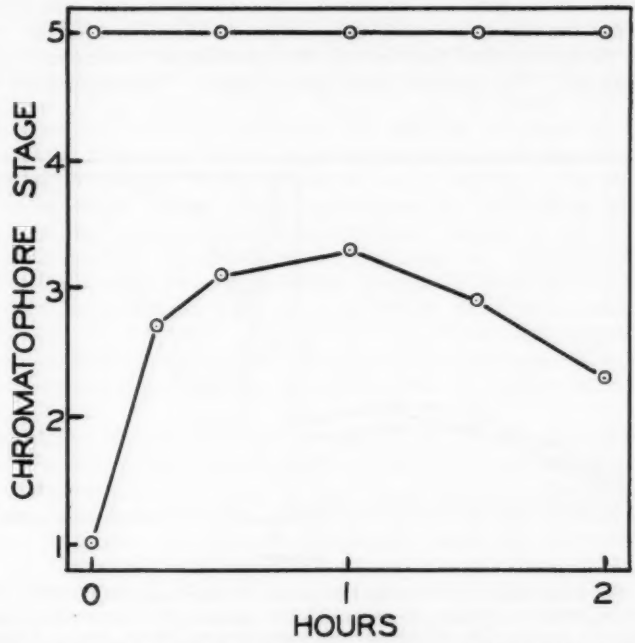

FIGURE 10. Responses of the red chromatophores of dwarf crayfish on black and on white backgrounds to epinephrine.

Responses of the red chromatophores of dwarf crayfish to epinephrine

An epinephrine solution (adrenalin chloride) manufactured by Parke, Davis and Co. was diluted with Van Harreveld's solution so that the final concentration was 2×10^{-5} gram epinephrine per 0.02 ml. Ten one-eyed dwarf crayfish with maximally concentrated red pigment and a like number with maximally dispersed red pigment were each injected with 0.02 ml. of the solution of epinephrine. The average stage of the red chromatophores of the crayfish in each pan was then determined 15, 30, 60, 90, and 120 minutes after they had been injected. The experiment was repeated once.

The results are presented in Figure 10 where each point represents the average of 20 crayfish. As is evident from inspection of the figure, epinephrine produced considerable pigment dispersion. No tendency for the red pigment to concentrate was apparent.

Filterability and adsorbability of the chromatophorotropic factors present in the supraesophageal ganglia and circumesophageal connectives of dwarf crayfish

An extract of the supraesophageal ganglia, with the circumesophageal connectives attached, of dwarf crayfish was prepared so that the final concentration was one-third of a complement per 0.02 ml. of fluid. The extract was then divided into three portions. One part was untreated. The second aliquot was filtered once through "Aloe Standard American" filter paper, catalogue no. 42700. The third was shaken through crushed charcoal and then centrifuged to remove the small particles of charcoal. The fractions were then assayed. The experiment was done twice.

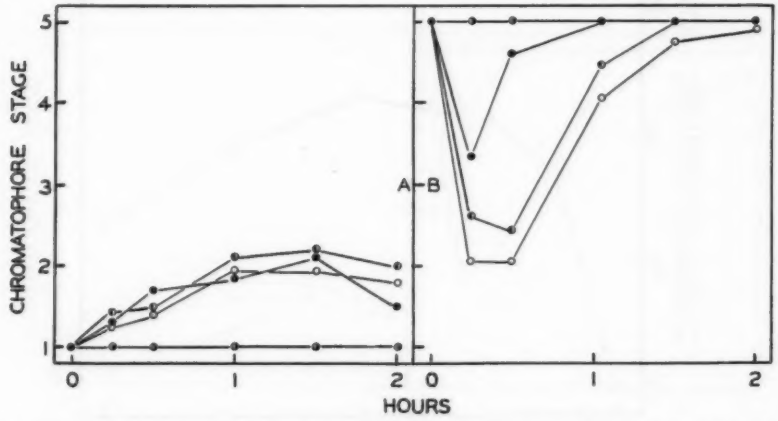

FIGURE 11. Responses of the red chromatophores of dwarf crayfish on white (A) and on black (B) backgrounds to extracts of supraesophageal ganglia, with the circumesophageal connectives attached. Circles filled on the left side, pure extract; circles, extract filtered through paper; dots, extract shaken with powdered charcoal; circles filled on right side, control.

The results are presented in Figure 11 where each point represents the average of 10 individuals. No striking differences were found among the titers of RPDH in the extracts. A slight adsorption of RPDH on filter paper may have occurred. In contrast, a striking decrease of RPCH in the extract shaken with charcoal was apparent whereas this substance was not adsorbed on filter paper. A slight augmentation of the titer of RPCH in the filtered extract was apparent although the reason for this is not clear since an appreciable quantity of its antagonist (RPDH) was still in the extract.

DISCUSSION

The experiments described above were based upon some problems that investigators of color changes in crustaceans have been considering for several years. One such is why an extract that concentrates pigment in the animal from which it

was obtained disperses pigment when injected into another species. This problem is strikingly exemplified by the results obtained when extracts of *Orconectes* and dwarf crayfish were injected into the donor species as well as interspecifically. Extracts of tissues of specimens of *Orconectes* never produced red pigment dispersion in specimens of *Orconectes* yet they produced striking concentration and dispersion of the pigment of dwarf crayfish. On the other hand, extracts of the eyestalk of dwarf crayfish were capable of producing dispersion of red pigment in specimens of both *Orconectes* and dwarf crayfish.

Specimens of *Orconectes* may have an excellent feed-back mechanism associated with the chromatophore system so that any displacement of the red pigment in crayfish on a white background to a more dispersed state is rapidly met with release of RPCH to maintain a steady-state of the red pigment. Extracts of the eyestalks of dwarf crayfish alone were able to overcome this mechanism because of the large amount of RPDH present in the extract relative to RPCH. The other extracts used had more RPCH present relative to the quantity of RPDH so that the *Orconectes* could easily antagonize the RPDH in these extracts by secretion of some RPCH. In support of this hypothesis are the results of Fingerman (1958) who showed that the blood of *Orconectes* always contained RPCH and RPDH and that the state of the pigment at any given time appeared to be due to the relative quantity of each hormone in the blood. He was able to produce slight dispersion of red pigment in specimens of *Orconectes* with blood from specimens on a white background and considerable dispersion with blood from *Orconectes* on a black background.

The conclusive evidence presented herein that the RPCH and RPDH found in the sinus gland are different from the materials found in the supraesophageal ganglia plus the circumesophageal connectives that concentrate and disperse red pigment adds another complication to the already complicated study of control of color changes in crustaceans. The problem immediately arises why an organism should produce two hormones to accomplish the same function. Since the maximal red pigment-dispersing effect obtained when extracts of eyestalks or sinus glands are used occurs sooner than that of the supraesophageal ganglia plus the circumesophageal connectives (Figs. 2 and 7), RPDH of the sinus gland may be used to move the pigment rapidly to the desired state of dispersion and once at this state the factor of the supraesophageal ganglia plus the circumesophageal connectives may take charge. Such a situation may explain the results of Fingerman and Lowe (1957b) who assayed the amount of RPDH in the blood of dwarf crayfish after a background change and found that the amount in the blood 30 minutes after transfer of crayfish from a white to a black background was more than needed to maintain the pigment in the maximally dispersed condition. Perhaps the excess titer was due to RPDH from the sinus gland and once the pigment had become maximally dispersed the pigment was kept in this state by a lower titer of RPDH from the supraesophageal ganglia and the circumesophageal connectives.

Fingerman (1956) demonstrated that in the blue crab, *Callinectes sapidus*, the factor in the sinus gland that dispersed red pigment was different from the hormone in the circumesophageal connectives that accomplished the same effect. Perhaps this difference in hormones in the sinus gland and central nervous organs is a general thing among crustaceans.

Carstam (1951) showed that an enzyme that inactivated the red pigment concentrating hormone of the sinus gland of the prawn *Leander adspersus* was present in the hypodermis of the following crustaceans, *Leander adspersus, Cancer pagurus, Homarus vulgaris,* and *Idothea neglecta.* Presumably inactivation of RPCH of *Cambarellus* (Fig. 9) was due to the presence of this enzyme in the hypodermis of the unboiled carapace.

Results obtained by different investigators with mammalian hormones injected into crustaceans do not form a consistent pattern. Östlund and Fänge (1956) found that epinephrine dispersed red pigment in *Leander adspersus.* The same results were obtained with dwarf crayfish (Fig. 10). In contrast, Nagano (1950) found that adrenalin produced pigment concentration in the shrimp *Paratya compressa* and Abramowitz and Abramowitz (1938) found no effect of adrenalin on the chromatophores of the fiddler crab *Uca pugilator.* McVay (1942) found adrenalin ineffective on the red chromatophores of the crayfish *Orconectes immunis.*

The results presented above showed that RPCH of the supraesophageal ganglia plus the circumesophageal connectives is not removed by filtering through paper but is adsorbed on charcoal. McVay (1942) found the same results with extracts of the central nervous system of *Orconectes immunis.* She postulated that RPCH was present to some extent in combination with a non-active carrier. Furthermore, she postulated that neither the complex nor the dissociated form of the hormone was adsorbed on filter paper but that the complex would be adsorbed by charcoal but not the dissociated form. This postulated complex might consist of hormone molecules within neurosecretory granules. Evidence in support of this concept has been obtained for the black pigment-dispersing hormone of the fiddler crab *Uca pugilator* by Pérez-González (1957).

Summary and Conclusions

1. Hormones that concentrated and dispersed red pigment were present in the eyestalks and supraesophageal ganglia plus the circumesophageal connectives of the crayfishes *Orconectes clypeatus* and *Cambarellus shufeldti.*

2. Direct injection of extracts of eyestalks and supraesophageal ganglia plus the circumesophageal connectives of *Orconectes* into *Orconectes* produced red pigment concentration alone, whereas the same extracts dispersed as well as concentrated red pigment in *Cambarellus.* Extracts of eyestalks of *Cambarellus,* however, dispersed red pigment in *Orconectes.*

3. Boiled extracts of supraesophageal ganglia plus the circumesophageal connectives produced more dispersion of red pigment than unboiled extracts.

4. Maintenance of boiled and unboiled extracts of supraesophageal ganglia plus circumesophageal connectives of *Cambarellus* for two hours at room temperature yielded additional information about the stability of chromatophorotropins. Red pigment-dispersing hormone in unboiled extracts increased and concentrating effect decreased. In contrast, in boiled extracts no tendency for red pigment-concentrating hormone to disappear was evident whereas dispersing hormone decreased. The red pigment-dispersing hormone is probably present in heat-labile neurosecretory granules as well as in the free state.

5. The hormones in the sinus gland that dispersed and concentrated red pigment were different from the factors in the supraesophageal ganglia plus the circumesophageal connectives with the same functions.

6. Evidence was presented for an enzyme in the hypodermis that inactivates chromatophorotropins.

7. Epinephrine dispersed red pigment in specimens of *Cambarellus*.

8. The results were discussed in relation to the data of other investigators.

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POTASSIUM AND SODIUM REGULATION IN AN INTERTIDAL CRAB

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This paper will show that when the crab *Pachygrapsus crassipes* is exposed to a simple osmotic stress it regulates its blood sodium and potassium equally well. However, more potassium than sodium is exchanged between animal and external medium for a given alteration in the blood, which means that a source of potassium other than the blood is contributing to the exchanges. Also, evidence will be produced indicating that sources other than the blood contribute to sodium exchanges between crab and medium, thus suggesting the presence of adaptive salt pools, a phenomenon postulated by Hukuda (1932).

Much work has been done, especially on mammals, demonstrating that the ionic concentration in tissues can be altered by experimentally varying the concentration of ions in the environmental fluid. Such studies are reviewed by Manery (1954) and Harris (1956). Krogh (1939), Prosser *et al.* (1950) and Beadle (1957) consider the subject of ionic and osmotic regulation in aquatic animals, but these reviews are concerned chiefly with changes in the blood which are effected by alterations in the concentration of the external medium. Little information is available concerning the fate of ions entering an aquatic animal from a hypertonic external medium, nor the source of ions leaving an animal to a hypotonic medium. Prosser *et al.* (1955) have demonstrated the final sodium and potassium blood concentration of the crab *Pachygrapsus crassipes* after it is exposed to diluted or concentrated sea water. However, the total exchange of these two ions between animal and medium has not been shown. Thus, it could not be stated, for example, whether the loss of blood ions by an animal to a hypotonic medium represented the total loss by the animal or whether sources other than the blood were contributing to the loss.

Materials and Methods

The shore crab, *Pachygrapsus crassipes* Randall, used in this investigation was collected at Newport and Laguna, California. *Pachygrapsus* is particularly suitable for this type of study because it can regulate osmotically in dilute as well as concentrated sea water. Also, it can live out of water for extended periods (Jones, 1941; Prosser *et al.*, 1955; and Gross, 1955). All specimens used were between molts and were mature, none weighing less than 20 grams.

Sodium and potassium concentrations were measured by means of a Beckman flame photometer using a standard which contained potassium and sodium approximating the respective proportions in the blood. Samples of blood of approximately 0.05 ml. were extracted serially from individual crabs, measured, in calibrated capillary tubes and diluted in 25 ml. of distilled water. Such samples were used

directly in the flame photometer. Known quantities of sodium added to the blood thus treated could be recovered within 2% with concentrations of about 500 mEq./l. and potassium could be recovered within 10% of concentrations of around 10 mEq./l.

The exchange of ions between animal and external medium was determined as follows: Blood from a crab recently removed from sea water of known concentration was analyzed for sodium and potassium. The same specimen was kept out of water for a brief time to permit the rapid coagulation of blood and then was immersed in a known volume of a different salinity having been rinsed first in that new salinity. The volume of medium varied from 50 ml. to 100 ml., depending on the size of the specimen. All animals could raise themselves out of the water and thus usually were not immersed completely. After a period of immersion (24–48 hours) during which time adequate precautions were taken against evaporation, the crab was removed, and its blood as well as external medium were analyzed again for potassium and sodium. Thus determinations of sodium and potassium were made on crabs both before and after exposure to the experimental media. The following experimental treatments were studied:

a) Transferred from normal sea water to dilute sea water (25% or 50%).
b) Transferred from normal sea water to concentrated sea water (approximately 150%).
c) Acclimated for 1–2 days to 50% sea water, then transferred to approximately 150% sea water.
d) Acclimated for 1–2 days to approximately 150% sea water, then transferred to 50% sea water.

The effects of desiccation on the blood concentrations of sodium and potassium also were investigated. Blood from crabs freshly removed from normal sea water was analyzed for potassium and sodium; the crab was then blotted dry, weighed and placed in a chamber at 15° C. for desiccation. After a period ranging from 24 to 72 hours, the animal was dipped in sea water to replace the evaporated branchial fluid, blotted and weighed. The blood then was analyzed again for sodium and potassium. Blood from a few partially desiccated crabs was analyzed for potassium and sodium; then the animal was desiccated further, and its weight change between the two desiccated conditions was measured without dipping as above, since there was little or no water remaining in the branchial chamber after partial desiccation. Then the blood was analyzed again for sodium and potassium. Since the effects of desiccation on the ionic concentration of the blood per unit weight loss by evaporation were not significantly different for the two above methods, it can be concluded that branchial fluid is accurately replaced by dipping and the weight losses caused by evaporation do not include the branchial fluid. Thus the blood sodium or potassium concentration change could be determined for a given weight loss caused by evaporation.

All of the above treatments were endured by most of the crabs tested which seemed to recover when returned to normal sea water. The repeated blood sampling resulting in a total loss of not more than 0.2 ml. does not seem to impose too great a stress since a 30-gram crab containing about 5 to 10 grams of blood can survive the loss of 1 ml.

Results

Table I, which presents the blood potassium and sodium concentrations of normal *Pachygrapsus*, freshly removed from the sea, shows the mean potassium concentration as 7.43 mEq./l. and the mean sodium concentration as 483.3 mEq./l. While the latter value is in close agreement with Prosser *et al.* (1955), the value for potassium is considerably less than 12.1 mEq./l. reported by the above workers.

Table I

Blood concentrations of crabs after treatment

Treatment		No. of crabs	Final blood concentration		Blood changes		
			Mean mEq./l.	S.D.	Mean mEq./l.	S.D.	% original
100% sea water to 25%* or 50% sea water	Na	28	373	37.0	−107	38.0	−22.8
	K	22	6.03	2.16	−2.25	2.11	−28.1
100% sea water to 150% sea water	Na	25	574	26.1	+99.2	26.1	+20.7
	K	24	9.71	1.54	+2.67	1.25	+38.3
50% sea water to 150% sea water	Na	12	572	37.2	+180	37.1	+43.5
	K	12	9.21	1.19	+3.26	0.90	+58.2
150% sea water to 50% sea water	Na	14	406	37.3	−174	61.2	−29.7
	K	14	6.78	1.35	−2.92	1.71	−29.2

		No. of crabs	Means		S.D.
Normal crabs freshly removed from the sea	Na	36	mEq./l.	483.3	17.3
			% sea water	103	
	K	36	mEq./l.	7.43	0.72
			% sea water	74	

* Blood concentrations of crabs immersed in 25% sea water were not significantly different from those immersed in 50% sea water. This is because the former were immersed for briefer periods.

This difference is even more significant because the potassium values obtained in the present investigation are less than those in the external medium (9.8 mEq./l.) whereas the values of Prosser *et al.* were more than the medium. The latter studies were made at Pacific Grove, California, about 300 miles north of the Laguna area where specimens for the present investigation were collected. Possibly temperature is the significant difference.

Not only is the blood potassium of *Pachygrapsus* less concentrated than the potassium of normal sea water, the natural medium of this crab, but it remains less concentrated even when immersed in media as dilute as 50% sea water, *i.e.*, there is a tendency for the blood potassium to remain less concentrated than that in the medium. For 28 crabs immersed 24–48 hours in 50% sea water (salts were lost from the animal, increasing the medium concentration) the mean ratio, blood potassium (mEq./l.)/medium potassium (mEq./l.) was 0.897, S.D = 0.19 which is significantly less than one, $P < 0.01$. As expected, the blood potassium is likewise less concentrated in hypertonic media.

Blood sodium on the other hand, which contributes about half of the blood osmotic pressure, was maintained above the sodium concentration of dilute media and below sodium concentrations in concentrated media, indicating active regulation of this ion as described by Prosser *et al.* (1955). Thus under the conditions of these experiments neither blood sodium nor potassium achieves concentrations equal to those of the external medium. Neither does the blood become isotonic to the external medium (Jones, 1941; Prosser *et al.*, 1955; Gross, 1957).

Table I also shows the ionic alterations that occur under various treatments in aqueous media. These values are presented to demonstrate the magnitude of blood ion changes, but they should not be considered comparable to those values reported in other investigations where animals were immersed completely in large volumes of water. The prime objective of the present investigation is to demonstrate the ionic change that occurs in the medium per given ionic change in the blood of the animal. It should be pointed out that the dilution and concentration of the blood of *Pachygrapsus* such as is shown in Table I is effected by salt exchange, not water (Gross, 1957). This must mean that a loss of ions in the external medium is essentially the same as an injection of salts into the animal. On the other hand a gain of ions in the external medium is the same as a removal of ions from the animal. We cannot say at this point whether or not those exchanges occur only between blood and external medium.

Now the "apparent volume of distribution" (Winkler *et al.*, 1943) in the animal for each ion can be estimated from the following equation,

$$V = M/P \times 100,$$

where: V = "apparent volume of distribution" in % body weight;

$$M = \frac{\text{weight of medium}}{\text{weight of animal}};$$

$$P = \frac{\text{change in blood ion concentration (mEq./l.)}}{\text{change in medium ion concentration (mEq./l.)}}$$

(the observed ratios, P are presented in column 2, Table V, corrected to an M value of 1.0).

Table II shows the effect of desiccation on the blood of crabs. After a crab is desiccated, the "apparent volume of distribution" can be estimated by the equation,

$$V = \frac{E}{C_1/C_2 - 1},$$

TABLE II

Sodium and potassium increases in the blood caused by desiccation

	No. of crabs	Change in ion concentration (% original concentration) per 1% body weight loss*		
		Mean	S.D.	95% fiducial limits
Na	84	+2.20	0.71	2.05– 2.35
K	50	+8.68	11.75	5.36–12.00

* By evaporation.

where $E = \%$ weight change caused by evaporation,

C_1 = initial blood ion concentration (mEq./l.),

C_2 = final blood ion concentration (mEq./l.).

Table III gives the "apparent volume of distribution" for sodium for the various treatments. Here it can be seen that the volume in question varies with the treatment. Thus by the desiccation method it averages 48.9% body weight. When crabs were transferred from normal sea water to dilute sea water the volume av-

TABLE III

"Apparent volume of distribution" for sodium

	Treatment	No. of crabs	Volume in % body weight	S.D.
A	Desiccation	84	48.9	12.8
B	Normal sea water to Dilute sea water	28	39.0	12.8
C	Normal sea water to Concentrated sea water	25	37.9	11.7
B + C	Normal sea water to Dilute or concentrated sea water	53	38.5	12.1
D	150% sea water to 50% sea water	14	46.9	4.52
E	50% sea water to 150% sea water	12	44.8	8.56
D + E	50% or 150% sea water to 150% or 50% sea water	26	45.9	6.55

erages 39.0%; where animals were transferred from normal sea water to concentrated sea water, 37.9% body weight. The latter two means are not significantly different from each other, so the values for the two treatments were combined, and these averaged 38.5% body weight. This value was shown by "t" evaluation to be significantly different from the above 48.9% value estimated by the desiccation method, $P = 0.003$. When large blood changes were effected by first acclimating the animal to either dilute or concentrated sea water (50 or 150%), then transferring it to the opposite stress and measuring the resultant changes in the blood

TABLE IV

Relative sodium and potassium concentration changes in the blood caused by osmotic stress

	Treatment	Blood sodium change (% original) / Blood potassium change (% original)		
		No. of crabs	Mean of ratios	S.D.
A	100% sea water to 25% or 50% sea water	22	1.24	1.31
B	100% sea water to 150% sea water	23	0.72	0.57
A + B	100% sea water to dilute or concentrated sea water	45	0.97	0.91
C	50% sea water to 150% sea water	12	0.83	0.31
D	150% sea water to 50% sea water	14	0.92	0.22
C + D	50% or 150% sea water to 150% or 50% sea water	26	0.88	0.26

and external medium of the animal, the "apparent volume of distribution" averaged 45.9% body weight. There was no significant difference between values calculated on crabs started in 50% sea water and those initially placed in 150% sea water. However, the volume 45.9% body weight was shown to be significantly different from 38.5% body weight determined for crabs transferred from normal sea water to various stresses (B + C), $P = 0.0004$. Yet, the "apparent volume of distribution" determined by (A), the desiccation method (48.9% body weight), was not significantly different from 45.9% body weight.

Table III reveals also that the variance in values for "apparent volume of distribution" is markedly reduced when the crab is exposed to extreme osmotic stresses

(*e.g.*, transferring from 50% to 150% sea water). This merely means that a large sodium change effected by the extreme stress can be measured with greater precision than a small sodium change. That is, the percentage error would be larger for the determination of a small change than for a large change since the accuracy of the method is constant. Thus the large variances observed for the desiccation method and for the moderate stress (B and C, Table III) method are believed to be the result of experimental error and not physiological variation.

The "apparent volume of distribution" for potassium in most cases was calculated to be greater than 100% body weight by the immersion method and averaged about 13% body weight by the desiccation method. It thus becomes clear that the "apparent volume of distribution" has little morphological significance even when referring to a specific ion. The following sections will show that differences in values for "apparent volume of distribution" are indications that sources of ions within the crab other than the blood are participating in exchanges with the medium.

First, in Table IV it can be seen that under various aqueous osmotic stresses the blood potassium changes of the crab are approximately equal to the blood sodium changes, percentage-wise. Of the four treatments represented in Table IV only in the case where crabs are transferred from normal sea water to concentrated sea water is the ratio, sodium change (% original)/potassium change (% original) significantly less than 1.0, $P = 0.05$. The other values are not significantly different from 1.0, which means that in general, potassium and sodium are regulated approximately equally in the blood of *Pachygrapsus* when the crab is subjected to osmotic stress. It should be observed that values in Table IV are means of individual ratios, blood sodium change (% original)/blood potassium change (% original), not ratios of the mean blood changes presented in Table I. Any discrepancy between the mean of ratios and the ratio of means can be explained by the observed variances.

Now let us assume that the mean "apparent volume of distribution" for sodium in crabs exposed to moderate stresses, 38.5% body weight, represents a constant volume of fluid in the animal in which both sodium and potassium concentrations are equal to those of the blood. This particular hypothetical volume, which hereafter shall be referred to as "A–D volume," was chosen because it was determined under conditions of moderate stress and would be expected to be closer to a possible morphological space than a value obtained under conditions of extreme stress. That is, a moderate stress is closer to a normal condition than is an extreme stress.

If then a change in the quantity of an ion in the external medium of an animal were known, by knowing the concentration change in the blood and the volume, 38.5% body weight, it could be determined what fraction of an ionic exchange between crab and external medium appears in the "A–D volume." Table V, column 1 reveals that in the case of potassium less than half of a loss or gain by the medium (42% maximum) is calculated to be accounted for in the "A–D volume." This means that a tissue potassium pool (probably the intra-cellular space) is participating in the exchanges. Also in the case where the crab is exposed to extreme osmotic stresses, only part of the sodium change in the medium (84%) appears in the "A–D volume," again an indication of a salt pool. It has already been shown that the sodium exchanges which occur under extreme stress are significantly different from those occurring under moderate stress (see discussion of data

in Table III). Now with respect to potassium there is no such trend with increased stress. However, the calculated ratio, change in "A–D volume"/change in medium with respect to potassium for crabs transferred from normal sea water to dilute sea water (0.22) is significantly less than 0.38, calculated for animals

TABLE V

Sodium and potassium exchanges between Pachgrapsus and ambient stress media

	Treatment		No. of crabs	(1) Calculated Change in "A-D volume" (mg.) / Change in medium (mg.)	(2) Observed Change in blood (mEq./l.) / Change in medium (mEq./l.)*	
				Mean	Mean	S.D.
A	100% sea water to 25% or 50% sea water	Na	28	0.98	2.56	0.82
		K	20	0.22	0.56	0.43
B	100% sea water to 150% sea water	Na	25	1.10	2.63	0.80
		K	24	0.38	0.998	0.70
A + B	100% sea water to dilute or concentrated sea water	Na	53	1.00**	2.60**	0.82
		K	44	0.31	0.80	0.62
C	50% sea water to 150% sea water	Na	12	0.86	2.23	0.43
		K	12	0.33	0.87	0.50
D	150% sea water to 50% sea water	Na	14	0.82	2.13	0.22
		K	14	0.42	1.08	0.35
C + D	50% or 150% sea water to 150% or 50% sea water	Na	26	0.84	2.18	0.32
		K	26	0.38	0.98	0.44

* Change in medium was caused by ions lost or gained by the crab. This change in all cases was corrected for a volume of medium equal to the weight of the crab because the ratio, weight of medium/weight of animal was not equal for all crabs.

** $$\frac{\text{"A-D volume"}}{\text{Medium volume}} \times \frac{\text{Change in blood (mEq./l.)}}{\text{Change in medium (mEq./l.)}} = \frac{\text{Change in "A-D volume" (mg.)}}{\text{Change in medium (mg.)}}$$

$= 0.385 \times 2.60 = 1.00.$

transferred from normal sea water to 150% sea water, $P = 0.01$, and also significantly less than 0.42, calculated for crabs treated by transferring from 150% sea water to 50% sea water, $P = 0.001$. On the other hand, 0.22 is not significantly different from the calculated value, 0.33, obtained for crabs treated by transferring from 50% sea water to 150% sea water. I have no explanation for this curious

fact that the ratio, change in "A–D volume"/change in medium for animals transferred from 100% sea water to dilute conditions, was lower than for some of the other treatments. The important conclusions that can be made from the data contained in Table V are: a) For a given change of the respective ions in the external medium more than twice as much sodium as potassium can be accounted for in the "A–D volume," which means that a potassium source other than the blood is participating in exchanges with the medium. b) The percentage of a sodium change in the medium which can be accounted for in the "A–D volume" decreases with increased osmotic stress, again suggesting the participation of a sodium pool. c) The salt pools quantitatively have the same role in hypertonic media as in hypotonic media.

Discussion

The validity of the above conclusions concerning the presence of salt pools does not depend on the validity of the value 38.5% body weight for "A–D volume." Qualitatively, the same conclusions can be reached from the data in column 2, Table V which are the *observed* ratios, change in blood (mEq./l.)/change in medium (mEq./l.) from which the *calculated* ratios, change in "A–D volume" (mg.)/change in medium (mg.) can be derived (column 1, Table V).

The different values for "apparent volume of distribution" (Table III) cannot be interpreted as a varying morphological space filled with fluid in which sodium is dissolved in concentrations equal to those of the blood. It has been established already (Gross, 1957) that *Pachygrapsus* does not gain or lose water significantly when immersed in an osmotic stress. Therefore, a sodium pool must be contributing to the exchanges which occur between animal and medium. It will be remembered that the values for "apparent volume of distribution" for potassium are usually more than 100% body weight. This suggests, of course, that the potassium of the intra-cellular fluid, known to be in relatively high concentrations, is participating in the exchanges with the medium, in effect acting as a potassium pool.

Now by assuming a constant volume of fluid in the crab ("A–D volume") in which sodium and potassium are dissolved in concentrations equal to those of the blood, we can arrive at a quantitative estimation of the role of the salt pool for a given ion exchange between animal and medium (Table V, column 1). Figure 1 illustrates further how a salt pool might function under conditions of *extreme* stress. Here a 100-gram crab whose "A–D volume" is 38.5% body weight is immersed in 100 ml of 50% or 150% sea water. Under conditions of presumed equilibrium this results in an assumed 20% alteration in the blood sodium; *i.e.*, from 500 mEq./l. to 400 mEq./l. when in the dilute medium, and from 500 mEq./l. to 600 mEq./l. in the concentrated medium. Since blood potassium is regulated approximately equally to blood sodium percentage-wise (Table IV), the blood potassium under the above conditions will also be altered 20%; *i.e.*, from 8.0 mEq./l. to 6.4 mEq./l. in the dilute medium and 8.0 mEq./l. to 9.6 mEq./l. in the concentrated medium.

Using the volume 38.5% body weight for "A–D volume," the above concentration changes can be converted to quantities of the two ions in milligrams. Also, knowing the volume of the external medium and the concentration change there (the ratios, blood change (mEq./l.)/medium change (mEq./l.) are presented in Table V, column 2) the ion loss or gains in the medium can be expressed in milli-

grams. Thus it can be seen in the diagram (Fig. 1) that while 106 mg. of sodium enter or leave the "A–D volume" (solid-lined arrow), a net change of only 89 mg. occurs in the "A–D volume," that is, 84% of the flux (see Table V, column 1, C + D). The remaining 17 mg. of sodium are fixed in the salt pool in the hypertonic medium or released from the salt pool in the hypotonic medium (dotted arrows), thus significantly contributing to the mechanism of maintaining ionic and osmotic homeostasis in the body fluids.

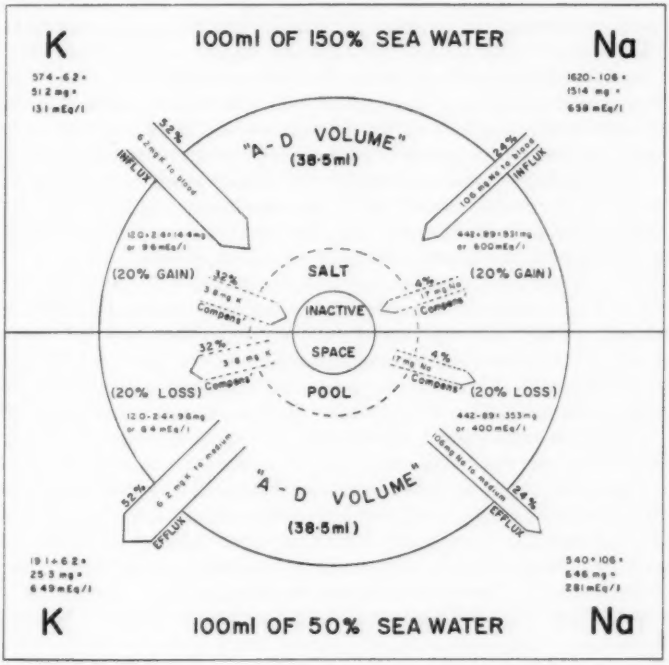

FIGURE 1. Suggested functional salt pool in a 100-gram crab immersed in osmotic stresses. Animal is represented by large circle; large square the external medium. Upper half of diagram illustrates net ion movements in 150% sea water; lower half; net ion movement in 50% sea water. Solid-line arrows indicate ion exchanges between external medium and "A–D volume" (assumed to be 38.5% body weight). Dotted arrows indicate ion exchanges between salt pool and "A–D volume." All percentages in diagram are with respect to initial blood concentrations (500mEq./l. for sodium; 8.0 mEq./l. for potassium). Numerical data are subject to small errors in rounding off. Compens' = compensation. The diagram may be read as follows: For example, for potassium loss in 50% sea water: a 100-gram crab assumed to have an "A–D volume" of 38.5% (explained in text) is found to lose 6.2 mg. of potassium to the medium, but its "A–D volume" potassium only goes down from 12.0 to 9.6 mg., a decrease of 2.4; the extra potassium is assumed to come from a pool and must be 6.2–2.4 = 3.8 mg. Therefore, the crab loses potassium equal to 52% of its initial "A–D volume" potassium, but the blood only decreases 20% and a compensation is calculated amounting to 32% of the initial potassium entering from the pool.

With respect to potassium, 6.2 mg. enter or leave the "A–D volume," but only a final change of 2.4 mg. remains in the "A–D volume," or about 38% of the flux (Table V, column 1). Again the remainder is fixed in or released from the salt pool.

It is possible that instead of compensatory exchanges occurring between salt pool and blood, salt fluxes occur directly between pool and external medium without passing into the "A–D volume." Such a phenomenon could yield the same results as presented in Table V, but it would seem negatively adaptive and also improbable because of the problem of transporting ions directly between pool and external medium through an exoskeleton which is relatively impermeable (Gross, 1957). It should be emphasized that such ion fluxes to and from the salt pool would be the same per unit blood change in 50% sea water as in 150% sea water. Passive transport from a pool containing the concentration of sodium and potassium permitting such fluxes would be extremely slow, especially through the exoskeleton. In the case of potassium, a greater net exchange occurs from the salt pool than from the "A–D volume" and presumably the blood which is separated from the external medium by tissues known to be permeable, *e.g.*, gills. While some flux of salts may occur directly between pool and medium, it seems more likely that the blood is traversed by the majority of the exchanged ions.

The events described above in Figure 1 have assumed conditions of extreme stress such as might occur by transferring an animal acclimated to 50% sea water into 150% sea water. The calculated role of the sodium pool in a lesser stress might decrease or become zero for the conditions in Figure 1 which were set up to explain the variation that occurs between two magnitudes of stress for the ratio, blood sodium change (mEq./l.)/medium sodium change (mEq./l.) (Table V, column 2). The morphological significance of "A–D volume" is obscure, but it seems possible that were the ratios in Table V, column 2, obtained under conditions of minute stress, the "apparent volume of distribution" for sodium would be smaller than 38.5% body weight and this smaller volume would have been chosen as the hypothetical constant "A–D volume." The principle would remain the same, however, namely that increased values for "apparent volume of distribution," for sodium with increased stresses does not indicate an increase in a volume of fluid, but rather participation of a sodium pool in the sodium exchanges between animal and medium.

The close agreement of values for sodium "apparent volume of distribution" obtained by the desiccation and the immersion method when animals were transferred from 50% sea water to 150% sea water or vice versa (extreme stress), is interpreted as a coincidence of values with possibly two different phenomena involved. Again, assuming the constant "A–D volume," 38.5% body weight, both the values, 45.9% body weight obtained from the extreme stress method and 48.9% body weight obtained from the desiccation method (Table III) can be explained as greater participation of sodium reservoirs. However, the value 48.9% body weight obtained by desiccation could also be explained by the participation of water pools which are inactive until conditions of desiccation exist when they are capable of replenishing water lost by evaporation. In either case, salt or water pool, the end result would be values for "apparent volume of distribution" which would be greater than those obtained under moderate immersion stresses (38.5% body

weight). In both cases there would be a tendency to maintain a constancy of blood which therefore would be an adaptive end result.

The large changes in the blood potassium relative to sodium that occur during desiccation (Table II) indicate that potassium passes from some sort of intrinsic supply into the blood. The inability of *Pachygrapsus* to regulate its blood potassium under conditions of desiccation may be an important factor limiting the terrestrial behavior of this crab. Gross (1955) discusses other limiting factors with respect to land habits of *Pachygrapsus*.

The nature of the above described salt pool may be merely the formed tissues responding to an osmotic or ionic stress, thus exchanging ions from the cytoplasm or cell surface when a gradient threshold is surpassed. This interpretation possibly is corroborated qualitatively by Shaw (1955) who demonstrated that muscle fibers of the crab *Carcinus* release both sodium and potassium when the animal is immersed in dilute sea water. Also, much information is available especially concerning mammals, demonstrating that the ionic concentrations of tissues can be altered by varying the concentration of ions in the environmental fluid. For example, muscle sodium will increase in an animal perfused with hypertonic sodium chloride solution; the liver shows gains in potassium when the plasma potassium is elevated, or muscle potassium will decrease if an animal is perfused with glucose solution. Such studies and similar studies concerned with isolated tissues are reviewed by Manery (1954) and Harris (1956).

It may well be that the findings of the present investigation are manifestations of the same general cellular mechanism, illustrated by the above mentioned perfusion experiments, *i.e.*, incidental ionic changes take place in the formed tissues in response to changes in the environmental fluid. However, it should be emphasized that *Pachygrapsus*, as an aquatic animal, must contend normally not only with salts and water reaching it by way of the gut but also with the flux of salts and water which occur continuously through permeable membranes separating the body fluids and tissues from the external medium, a problem not presented to terrestrial animals. It is interesting that in *Pachygrapsus* more than twice as much potassium is estimated to exchange between external medium and the crab, than the net exchanges calculated to occur in the "A–D volume" or probably the blood, itself (Fig. 1). Yet comparing the final blood concentrations with normal blood concentrations (Table I), the blood potassium does not vary from normal more than 30%. Thus, it seems that there is a tendency to maintain the blood potassium at a constant level at the expense of the tissue or pool potassium. This suggests a method of ionic regulation in the blood without need of a special organ such as a kidney.

It is possible, then, that the above described salt pools could have a special, functional significance which would be adaptive for an aquatic animal such as a crab. Thus, normally, osmotic and ionic constancy of the blood could be maintained at least partially by salt pools which are capable of mobilizing or fixing salts to and from the blood as the situation demands. Such a device would be necessarily of temporary value only, but would be particularly advantageous for estuarine forms which could make up an osmotic deficit from their salt pools at low tide and low salinities, then replenish the pools with a minimum of work when the salinity was elevated on the in-coming tide.

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Summary

1. When *Pachygrapsus* is immersed in a stress medium its blood concentration is altered by a loss of ions to a hypotonic medium and a gain of ions from a hypertonic medium. Water exchanges are insignificant in magnitude.

2. The observed ratio, change in blood ions/change in medium ions yields values for "apparent volume of distribution" for the respective ions. Such values vary according to the treatment for sodium and in moderate stresses average 38.5% body weight, in extreme stresses 45.9% body weight. For potassium most values came to more than 100% body weight and do not vary with increased stress. The above ratios are the same for a hypotonic medium as for a hypertonic medium.

3. Varying values for "apparent volume of distribution" under different magnitudes of osmotic stress suggest the presence of salt pools which may represent incidental participation of the formed tissues, or may represent an adaptive mechanism which functions to assist in the ionic and osmotic regulatory mechanism.

4. In stress media blood potassium and sodium are regulated equally well, percentage-wise, but a source other than the blood participates in the exchanges of potassium between animal and medium. Thus the potassium change in the blood does not account for the total potassium change in the animal.

5. "Apparent volume of distribution" calculated from the increased blood concentration caused by a given water loss by evaporation averages 48.9% body weight for sodium and only 13% body weight for potassium. The blood potassium therefore, increases percentage-wise about four times more than blood sodium. This indicates that potassium leaves a pool (probably the intra-cellular space) to enter the blood. This appears to be a physiological failure rather than regulation, and may play a role in ecological limitations.

6. Potassium concentrations in the blood of normal crabs (*Pachygrapsus*) are less than those of sea water. When immersed in dilute sea water of lower potassium concentrations than found in the blood of normal animals, the crabs usually tend to lose potassium so that it remains less concentrated than the potassium of the medium.

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A RE-EXAMINATION OF THE OSMOTIC PROPERTIES OF THE PACIFIC HAGFISH, POLISTOTREMA STOUTI

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Investigations of various constituents of the blood of vertebrates have led to generalizations about vertebrate origins and relationships. The entirely marine myxinoids or hagfishes are morphologically primitive in the vertebrate assemblage and have unique osmotic and ionic properties. Most authors have concluded that hagfishes are approximately isotonic to their environment and that the osmotic concentration of the blood is made up largely of electrolytes. In some instances results have shown either a slight or marked hypertonicity of the blood serum with respect to the environment (summary in Table I), in contrast to the general conclusion of isotonicity.

Dekhuyzen (1904), Greene (1904) and the Schmidt-Nielsens (1923) concluded from their measurements of the freezing-point depressions of the blood sera and external medium that hagfishes must be isotonic. Smith (1932) interpreted his results, and those of Dekhuyzen and of Greene, however, to indicate a slight hypertonicity. Borei (1935), in contrast, concluded that *Myxine* was distinctly hypotonic to its environment. Krogh (1939, p. 119) considered that the blood of hagfishes is in almost complete osmotic equilibrium with sea water. In the most recent review, Black (1957, p. 187) concurs with Krogh, but her Figure 4 suggests that hagfishes are slightly hypertonic to sea water.

In view of the osmotic uniqueness of hagfishes it is important to know whether they are isotonic or are hypertonic to their environment. In the present investigation we have undertaken to determine whether the blood of the Pacific hagfish, *Polistotrema stouti*, is isotonic or hypertonic to the external medium over a range of concentrations. The chloride and sodium concentration of the blood serum and of the external environment were determined for comparison with previous work (see Table I). Since some of the variation of previous results may have been caused by different methods of handling and analysis, our own methods are described fully.

Materials and Methods

1. *Capture of animals*

On May 14, 1957, *Polistotrema stouti* (Lockington) was trapped by one of us (WNMcF) near San Diego, California. The trap was constructed from a 5-gallon can (MacGinitie and MacGinitie, 1949, p. 415), baited with two dead mackerel and set for 7 hours in 150 fathoms of water 4 miles north of North Coronado Island, Mexico. Of approximately 100 hagfish caught, 45 were transported to the laboratory at Marineland of the Pacific, Palos Verdes, California.

2. Care of animals

The hagfish were kept in three-gallon jars that were submerged in sea water in a large refrigerated tank (11–14° C.). Aerators were inserted through holes in the jar lids. There were no deaths under these conditions during a two-month period. Food was not offered until after the conclusion of the experiment performed on May 27. Dead mackerel were then placed in the jars on several occasions. Feeding was infrequent and the viscera only were consumed.

TABLE I

Previous measurements of osmotic pressure and chloride concentration of hagfish blood

Species	External medium		Internal medium		Internal medium[b] / External medium	Urea (mM/l.)	Source
	Δ_e[a] (° C.)	Chloride (milli-eq./l.)	Δ_i[a] (° C.)	Chloride (milli-eq./l.)			
M. glutinosa	1.73	—	1.74 1.83	—	Isotonic	—	Dekhuyzen (1904)
P. stouti	1.92	—	1.97	—	Isotonic	—	Greene (1904)
M. glutinosa	0.97 1.26 1.85 2.32	—	1.25 1.40 1.85 2.32	—	Isotonic	—	Schmidt-Nielsens (1923)
M. glutinosa	1.88	—	1.85 1.93 1.98	465 to 476	Hypertonic	2–4	Smith (1932)
P. stouti	—	384 467 530 626	—	344 414 471 570	Isotonic $Cl_i/Cl_e = 0.89$	Not detected	Bond, Cary and Hutchinson (1932)
M. glutinosa	1.90[e]	520	1.50[e]	325[d]	Hypotonic $Cl_i/Cl_e = 0.62$	58–62	Borei (1935)
M. glutinosa	—	483	—	448	Isotonic $Cl_i/Cl_e = 0.93$	—	Cole (1940)[e]
M. glutinosa	—	592[f]	—	576[f]	Isotonic $Cl_i/Cl_e = 0.91$[f]	2–4	Robertson (1954)

[a] Subscript e = external; i = internal.

[b] Interpretation of original author.

[c] Calculated from chloride and urea concentrations.

[d] Analysis of whole blood; low value may be related to low chloride concentration of red blood cells (see Robertson, 1954).

[e] Data of Smith, obtained in 1927.

[f] Chloride values are millieq./kg. water; ratio has been converted so that it is directly comparable to the others.

3. *Adjustment of animals to sea water of three different concentrations*

In addition to natural sea water (100%) a dilute medium of approximately 85% sea water was obtained by mixing 100% sea water and distilled water. Sea water was concentrated by boiling to half the original volume. This was mixed with natural sea water to provide a medium of approximately 115% sea water. The precise concentrations were obtained from freezing-point measurements. Hagfish were transferred in the three-gallon jars directly to sea water of these three concentrations. After an adjustment period of 30 hours, in which temperature was controlled by partially immersing the jars in the refrigerated tank, the hagfish were transported in these vessels to the University of California, Los Angeles, where the sampling was performed. Upon arrival the jars were placed in a cold room (2° C.) for a period of 1–3 hours. During this time the water temperature declined from 11.0° C. to a low value of 6.5° C. These temperatures are within the normal temperature range hagfish encounter.

4. *Blood sampling*

To facilitate withdrawal of blood samples the hagfish were stretched on a board with hemostats. Blood was removed in a syringe from the subcutaneous sinus. The freezing point of the first 0.2 ml. of whole blood was determined while more blood was withdrawn from the animal. Difficulty in obtaining blood from the thoracic or caudal hearts or from the systemic blood vessels made use of the sinus necessary.

The subcutaneous sinus of hagfishes is part of the blood circulatory system and not part of the lymphatic system (Cole, 1926, p. 322). It is one of a large system of sinuses which in cyclostomes appear to partially replace capillary beds. Blood flows into this extensive sinus from arteries of the snout and slowly moves posteriorly to the tip of the tail, where it is collected in a special vessel and pumped into the systemic circulation by the caudal hearts.

After withdrawal blood was placed in a 12-ml. centrifuge tube. A preliminary trial had shown that centrifugation of uncovered blood samples concentrated the blood sera by evaporation. The blood samples were therefore covered with paraffin oil immediately after withdrawal. No anticoagulant was necessary. Following centrifugation at 3500 times gravity for 20 minutes to remove cellular elements, the yellow or reddish supernatant fluid was drawn from beneath the paraffin oil in a slender-tipped pipette and placed in another centrifuge tube. Samples for chloride and sodium ion determination were removed and again a paraffin oil cover was added. The blood sampling and whole-blood freezing-point determinations were performed at the same time; freezing points of the serum were measured within the next 24 hours. The covered serum samples were stored in a refrigerator at 5° C.

The total length, weight and sex were determined for each specimen following blood removal. Specimens ranged in total length from 300 mm. to 460 mm. Their weights varied from 41.4 gm. to 135.3 gm.

5. *Freezing-point determinations*

Freezing points were measured with the Fiske Osmometer, which employs a thermistor. Samples of 0.2 ml. were placed in the small sample adapter of this

instrument and rapidly supercooled in the propylene glycol bath ($-10°$ C.). Vibration of a wire initiated freezing of the sample; resistance of the thermistor element, which was located in the freezing mixture, was balanced with a variable resistance. The osmometer had been previously calibrated with standard NaCl solutions; individual determinations of single samples varied no more than $\pm 0.02°$ C. Just before withdrawal of the hagfish from each container, freezing points of a water sample from that container were determined three times and the mean of these values recorded. Whole-blood and serum samples were determined only once to prevent denaturation of the protein fraction from changing the osmotic pressure of the frozen and thawed samples.

6. *Chloride and sodium analysis*

The chloride concentrations of the medium and serum were determined by a modification of the Volhard method reported by Keys (1937) and Consolazio, Johnson and Marek (1951). The small volume of blood obtained did not allow replicate determinations; each result therefore represents one titration. A mean error of 3.3% was obtained in analyses of standard NaCl soluitons. Chloride values are reported in milliequivalents per liter. Sodium was determined with flame photometry by the direct method. A Beckman Model B spectrophotometer with flame attachment was used in this analysis. A standard curve was established from a solution containing sodium, potassium, calcium and magnesium in the proportions reported for these elements in sea water of 19 parts per thousand chlorinity (Sverdrup, Johnson and Fleming, 1942, p. 186). No special provision was made to remove proteins prior to analysis, nor was any agent, such as isopropyl alcohol, used in the diluting solution to aid in ignition. The mean error determined was 3.2%.

Results

Osmotic data

Experiment 1

Jars of 85.5%, 100%, and 116.1% sea water (percentages based on freezing-point determinations), each containing three hagfish which had been kept at these concentrations for 30 hours, were brought to the laboratory on May 20, 1957. Freezing-point depressions of the external medium and of whole blood and serum samples of each hagfish were measured (Table II). Initially blood was drawn from one animal at each concentration (specimens 1D, 1N and 1C, Table II) and the osmotic pressure determined. The second animals tested at each sea water concentration (2D, 2N and 2C) had higher osmotic pressures. One hour was allowed to pass before specimens 3D, 3N, and 3C were removed and the blood samples obtained. The osmotic pressure of these last three hagfish returned to values near the concentrations of their respective environments.

This experiment indicated that *Polistotrema stouti* becomes isotonic or slightly hypertonic to its environment over the range of salinities from $\Delta = 1.59$ to $2.16°$ C. The Δ's of serum and whole blood were almost the same (ratio of Δ_s/Δ_{wb} from 99.4% to 102.3%). The slight hypertonicity of whole blood and serum to the external medium appeared to increase during the experiment, but after an hour the

blood returned toward isotonicity with the environment. These changes were quite uniform throughout the range of salinities tested.

Experiment 2

A second experiment was performed on May 27 with sea water at 4 concentrations from 83.5% ($\Delta = 1.57°$ C.) to 116.0% ($\Delta = 2.18°$ C.). Only a single hagfish was kept in each jar. The freezing point of the sea water in each container was measured just before removal of the hagfish.

TABLE II

Freezing-point depressions of whole blood (Δ_{wb}) and serum (Δ_s) of hagfish which had been kept in 3 different concentrations (Δ_e) of sea water for 30 hours. D, N, and C refer to dilute (85%), normal (100%) and concentrated (116%) media

	Sea water concentration (%)	Δ_e (° C.)	Animal number	Δ_{wb} (° C.)	Δ_s (° C.)	$\frac{\Delta_{wb}}{\Delta_e}$ (%)	$\frac{\Delta_s}{\Delta_e}$ (%)	$\frac{\Delta_s}{\Delta_{wb}}$ (%)
Experiment 1	85.5	1.59	1D	1.59	1.58	100.0	99.4	99.4
			2D	1.64	1.65	103.1	103.8	100.6
			3D[a]	1.61	1.60	101.3	100.6	99.4
	100.0	1.86	1N	1.87	1.89	100.5	101.6	101.1
			2N	1.93	1.94	103.8	104.3	100.5
			3N[a]	1.88	1.89	101.1	101.6	100.5
	116.1	2.16	1C	2.21	2.21	102.3	102.3	100.0
			2C	2.22	2.27	102.8	105.1	102.3
			3C[a]	2.19	2.23	101.4	103.2	101.8
Experiment 2[b]	84.6	1.59	4D	1.59	1.59	100.0	100.0	100.0
	83.5	1.57	5D	1.58	1.57	100.6	100.0	99.4
	100.0	1.88	4N	1.89	1.90	100.5	101.1	100.5
	100.0	1.88	5N	1.91	1.88	101.6	100.0	98.4
	105.9	1.99	4C	1.96	2.00	98.5	100.5	102.0
	116.0	2.18	5C	2.19	2.22	100.5	101.8	101.4

[a] A one-hour wait intervened before blood samples were drawn from the last animal in each container.

[b] Each hagfish kept in separate container.

The results (Table II) leave no doubt that the hagfish under these conditions were isotonic, not hypertonic, to the external medium (Δ_e between 1.57 and 2.18° C.). The Δ of 1.99° C. in one of the jars of concentrated sea water was the result of an unintentional dilution with 100% sea water during the preliminary adjustment period. The individual at this concentration, however, matched its osmotic environment about as well as the others.

In both experiments animals at different concentrations showed perceptible differences in the available volume and the apparent viscosity of blood. Those in diluted sea water provided a greater quantity of more "watery" blood than normal.

In concentrated sea water smaller amounts of more viscous blood were obtained. The external appearance of the animals did not differ appreciably in the three sea water concentrations.

Ionic data

The serum chloride and sodium concentrations corresponding to the freezing points reported in Experiments 1 and 2 are listed in Table III. Like the freezing-point values, the serum chlorides of the hagfish in Experiment 1 also show an

TABLE III

Sodium and chloride concentrations of the serum of Polistotrema stouti in Experiments 1 and 2

	Animal number	Total concentration[a] (milliosmols)		Milliequivalents/liter			
		Medium	Blood serum	Cl_e	Cl_i	Na_e	Na_i
Experiment 1	1D	853	849	441	419	330	—
	2D	853	886	441	431	330	360
	3D	853	861	441	385	330	335
	1N	999	1018	514	490	480	450
	2N	999	1043	514	516	—	—
	3N	999	1015	514	487	480	405
	1C	1162	1187	619	549	590	520
	2C	1162	1220	619	538	590	520
	3C	1162	1200	619	472	—	—
Experiment 2	4D	855	855	449	370	360	415
	5D	847	845	450	435	—	—
	4N	1010	1023	469	431	390	430
	5N	1010	1012	480	492	—	—
	4C[b]	1071	1077	530	502	405	455
	5C	1172	1193	576	529	570	475

[a] Determined from freezing-point depression.
[b] 106% sea water.

increase in concentration in the second animals tested (specimens 2D, 2N, and 2C) at each concentration. One hour later the hagfish (3D, 3N, 3C) showed a decline in serum chloride toward or below the original value. Where data were obtained, the sodium concentrations of sera in Experiment 1 also exhibit this trend.

DISCUSSION

From the experiments described above it is clear that the Pacific hagfish, *Polistotrema stouti*, is isotonic to the external medium, given time (30 hours in these experiments) to adjust. A slight apparent hypertonicity (no more than 5%) which developed during the course of Experiment 1 was eliminated in Ex-

periment 2 by keeping the hagfish separate. In Experiment 2 the average of $\Delta_{wb}/\Delta_e = 100.3\%$ and of $\Delta_s/\Delta_e = 100.7\%$. These values indicate complete isotonicity, within experimental error of the methods used.

Comparison of Experiments 1 and 2 offers a possible explanation for some of the differences reported by earlier workers. In handling the hagfish prior to withdrawal of blood it was impossible to prevent them from secreting slime copiously. In Experiment 1 removal of the first animal from each jar disturbed the others and caused sliming. The blood of the second animal from each medium was more concentrated than the first, but after an hour's wait the third animal from each medium was nearly isotonic. It seemed possible, therefore, that sliming induced a temporary hypertonicity of the blood. Support for this hypothesis was obtained in a subsequent experiment, in which as many as four small blood samples were withdrawn from single individuals at different times. Initial blood samples indicated isotonicity with sea water; production of slime was followed by hypertonicity (freezing-point measurements of whole blood samples) of 1–3%, which lasted an

TABLE IV

The contribution of sodium and chloride ions to the osmotic pressure of Polistotrema stouti. Ratios are mean values computed from the data of Table III. Parentheses denote the number of determinations from which each mean was computed

Concentration of medium (%)	Cl_i/Cl_e	Na_i/Na_e	$\frac{Na_e + Cl_e}{\text{milliosmols}_e}$[a]	$\frac{Na_i + Cl_i}{\text{milliosmols}_i}$[a]
85	0.918(5)	1.088(3)	0.914(4)	0.882(3)
100	0.970(5)	0.952(3)	0.946(3)	0.881(3)
116	0.874(5)	0.914(4)	0.985(4)	0.874(4)
Grand mean	0.918(15)	0.965(10)	0.953(11)	0.878(10)

[a] Determined from freezing-point depression.

hour or more. Much greater increases in the blood concentration were induced by rough handling or by placing the hagfish in a dry atmosphere. After a combination of these treatments for 10 minutes, the blood became 15–25% hypertonic to sea water. Osmotic recovery was nearly complete within 24 hours. The slight hypertonicity of hagfish blood indicated in the results of Dekhuyzen (1904), Greene (1904) and Smith (1932) may have been caused by handling or the secretion of slime. This could not account for the hypotonic values calculated by Borei (1935).

Under conditions of the present experiments hagfish become isotonic to 84–116% sea water ($\Delta_e = 1.57$ to 2.18° C.). The limits of their ability to endure changed external concentrations were not tested and it is not clear whether they might be able to regulate their osmotic concentration in some range other than 84–116% sea water. An insufficient number of hagfish was obtained to investigate this problem; work will be continued as more animals become available. The mean value (15 animals) of Cl_i/Cl_e was 0.92, which is similar to reports of most other authors (see Table I). In Robertson's paper (1954) on *Myxine* the sum of Cl_i and Na_i values accounted for 95.9% of the total osmotic concentration of the blood. In the present work this ratio was 95.3%, showing the close agree-

ment of our results with his. For computation of this ratio the grand mean of $Na_i + Cl_i$/milliosmols$_i$ (Table IV) was divided by 0.921, the factor given by Robertson to convert molar to molal concentrations in *Myxine*.

About 90% of the total osmotic concentration of the serum is due to sodium and chloride ions (Table IV); Robertson (1954) showed that other ionic constituents account for most of the remaining concentration. Both he and Smith (1932) found that there were 2–4 mM/l. of urea in the blood of *Myxine*. The high values of 58–62 mM/l. obtained by Borei (1935) are probably incorrect (nonspecific tests were performed on whole blood samples; see Robertson, 1954). In a personal communication Dr. Ernest Baldwin informs us that he has not been able to detect urea in the liver of *Polistotrema*. He points out that there is little likelihood that it would occur elsewhere in the body when absent from the liver. Dr. Baldwin, like Black (1957, p. 182), suggests that the nature of the food would affect the urea content of the tissues. It is possible that even the very low concentrations of urea reported by Robertson and Smith had an exogenous origin. Urea cannot have an importance in the osmotic composition of hagfishes comparable to that which it has in elasmobranchs.

Although the hagfishes resemble other vertebrates in the ratios of the ionic constituents of their blood (Cole, 1940; Robertson, 1954), they are unique among vertebrates in being isotonic to sea water and having the osmotic concentration of the blood composed almost entirely of ionic constituents. Hagfishes are poikilosmotic within the range of sea water concentrations investigated, which encompass those of the marine habitats they normally occupy.

We wish to acknowledge the assistance of Drs. David Jensen and Arthur Kelly in obtaining the specimens, as well as the University of California at La Jolla for use of the vessel *T-441*. Dr. Ernest Baldwin has kindly allowed us to use an unpublished chemical analysis of hagfish liver. Mr. Harry Hansen of the Long Beach Water Treatment Plant made available a flame photometer and assisted in the sodium analyses. To Drs. Boyd W. Walker and Frederick Crescitelli go our thanks for their interest and suggestions during the investigation. Mr. Richard Rosenblatt read the manuscript critically.

Summary

1. Determinations of the osmotic pressure of whole blood and serum show that *Polistotrema stouti* is isotonic in sea water concentrations from 85 to 116%.

2. Hypertonicity of the blood can be induced experimentally by disturbance of the animals. This factor could account for hypertonic values reported previously.

3. Serum sodium and chloride account for 88% of the total osmotic pressure. The mean Cl_i/Cl_e ratio equals 0.92. The hypertonicity which can be produced by disturbance is reflected by a rise in the serum sodium and chloride concentrations.

4. Urea is absent from the liver and is considered to have no significance in the osmotic composition.

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THE ACOUSTICAL BEHAVIOR OF SOME FISHES IN THE BIMINI AREA [1, 2]

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While many kinds of marine organisms are recognized as sound sources, it is not clear in most cases under what conditions the species concerned became sonic in nature, nor what patterns of behavior the sounds normally accompany. Spontaneous sound production, by many fishes known to produce sounds, tends to become partially or completely suppressed in captivity (Dobrin, 1947; Fish, 1948; Moulton, 1956b); one must frequently resort to artificial stimuli such as handling or electric shock (Fish, 1954; Fish *et al.*, 1952) to elicit any sound for study. Failure of free fishes to respond consistently to man-made sounds introduced into the water (Moulton and Backus, 1955) has aroused considerable interest, evident in writings since the time of Aristotle, in the role that the sounds of fishes themselves play in nature.

In order to obtain further information on the production of fish sounds and their significance to fish behavior, the period from 13 June to 13 August 1956 was spent at the Lerner Marine Laboratory of the American Museum of Natural History on North Bimini, Bahama Islands. A glass-panelled power boat and the clarity of the water facilitated extensive observation of near-shore fishes during underwater listening, and well-stocked fish pens provided opportunity for close observation of such fishes in a reasonably natural environment. Recordings of marine sounds, accompanied by notes on simultaneous observations, have been compared with recordings made in the laboratory from carefully identified specimens. The identity of certain sounds recorded at sea and in the laboratory has been determined by this aural comparison and by the study of vibragrams made at the Woods Hole Oceanographic Institution.

The fishes most frequently heard along the western edge of the Great Bahama Bank in the Bimini area, the Nassau grouper, *Epinephalus striatus* (Bloch), and the squirrelfish, *Holocentrus ascensionis* (Osbeck), are inhabitants of relatively shallow water. That sightings of the Nassau grouper and squirrelfish occurred only in areas where incidence of their calling was high, and that these species were generally observed in areas where their sounds were recorded, indicates a value of underwater listening in studying the distributions, even limited ones, of some sound-producing species.

Listening equipment used in the investigation consisted of an AX-58-C Rochelle salt hydrophone and a Woods Hole Suitcase amplifier; an undesignated Rochelle

[1] Contribution No. 957 from the Woods Hole Oceanographic Institution.

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TABLE I

Fishes making no sounds or only chewing sounds during the Bimini study

Family	Species	Comment
Acanthyridae	*Acanthus coeruleus*	1 specimen
Antennariidae	*Histrio histrio*	1 specimen
Apogonidae	*Apogon maculatus*	1 specimen
Balistidae	*Canthidermis sabaco*	Several individuals
Belonidae	*Strongylura marinus*	1 specimen
Chaetodontidae	*Chaetodon striatus*	1 specimen
	Pomacanthus paru	A 4" specimen
Clupeidae	*Jenkinsia lamprotaenia*	Large school and individual specimens
Echeneididae	*Phtheirichthyes lineatus*	2 specimens
Fierasferidae	*Carapus affinis*	1 specimen
Gerridae	Unidentified majarra	Several specimens
Gobiidae	*Bathygobius soporator*	Feeding sounds recorded from a group. Male known to call in the breeding season (Tavolga, 1956).
Haemulidae	*Haemulon album*	Several specimens
	Haemulon parra	Several specimens
Labridae	*Halichoeres radiatus*	Feeding sounds recorded
Lutianidae	*Lutianus griseus*	1 specimen
Malacanthidae	*Malacanthus plumeri*	1 specimen
Mullidae	*Upeneus maculatus*	Feeding sounds recorded
Ogocephalidae	*Ogocephalus radiatus*	1 specimen
	Ogocephalus vespertilio	1 specimen
Orectolobidae	*Ginglymostoma cirratum*	1 specimen
Pristidae	*Pristis pectinatus*	1 specimen
Rachycentridae	*Rachycentron canadus*	1 specimen
Scorpaenidae	*Scorpaena plumeri*	1 specimen
Serranidae	*Promicrops itaiara*	1 specimen
Sparidae	*Calamus providens*	1 specimen
Sphyraenidae	*Sphyraena barracuda*	1 specimen

salt hydrophone and a modified Heathkit amplifier Model A-7C, or an Ekotape microphone Model 205. Recordings were made at 7½ and 3¾ in./sec. on the Ekotape tape recorder and selected recordings have been analyzed on a vibration frequency analyzer, the Kay Vibralyzer. Sound-generating equipment used during the investigation consisted of a Hewlett-Packard audio oscillator Model LAJ or the Ekotape tape recorder, a Craftsman C550 amplifier, and a QBG transducer. All observations at sea were made in calm weather from the 30-foot motor launch RESEARCH of the Lerner Marine Laboratory, between 0800 and 1400 hours from 9 July to 12 August. Suitcase amplifier settings varied from $0 + 14 - 20$ to $0 + 10 - 5$. During work at sea, the hydrophone was suspended just above the bottom, or so that it cleared the bottom in the shoalest water in the listening area at drift stations.

Forty species of fishes, distributed among 29 families, were studied at Bimini

(Tables I and II). The stimuli used in eliciting sound in the laboratory were those of handling, capture in a net or feeding, or a combination of these. All sounds reported were recorded from isolated, submerged specimens, except the pectoral fin drumming and tooth stridulation of individual triggerfishes (Balistidae) which were not heard while the fish were being handled under water. All other sounds described were produced in air as well as under water, except the snap of the demoiselle, *Pomacentrus leucosticus* (Mueller and Troschel), which was recorded only from submerged specimens.

The species which produced no detectable sounds other than those of chewing

TABLE II

Characteristics of fish sounds recorded at Bimini

Family	Species	Vibration frequency analysis
Balistidae	*Balistes vetula* in air at microphone	Sound: Toothplate stridulation Duration: .12 sec./stridulation Frequency span: 0 to above 8 kc. Predominant intensities: .7–1.8 kc., 2.1–3.8 kc.
	Melichthys piceus in air at microphone	Sound: Toothplate stridulation Duration: .06 to .1 sec./stridulation Freq. span: 0 to above 8 kc. Pred. int.: 1.2 to 2.3 kc.
	B. vetula in air at microphone	Sound: Pectoral fin-drumming Duration: .03 sec./pulse Freq. span: 0 to 5.8 kc. Pred. int.: 1 to 1.7 kc.
	M. piceus in air at microphone	Sound: Pectoral fin-drumming Duration: .02 to .04 sec./pulse Freq. span: 0 to above 8 kc. Pred. int.: .7 to 2.2 kc.
	Feeding of a mixed group of the two spp. at AX-58-C hydrophone	Freq. span: 0 to above 8 kc. Pred. int.; .6 to 2.9 kc.
Carangidae	*Caranx hippos* hand-held in aquarium at hydrophone	Sound: Stridulation, pharyngeal teeth Duration: .06 sec./stridulation Freq. span: 0 to above 8 kc. Pred. int.: .3 to 1.2 kc., 1.7 to 3.3 kc.
Chaetodontidae	*Angelichthyes ciliaris* in cement tank at AX-58-C hydrophone	Sound: Grunt, single or repeated Duration: .06 to .1 sec./grunt Freq. span: 0 to 1.1 kc. Pred. int.: Below .5 kc.
	Pomacanthus arcuatus W. of Turtle Rocks at AX-58-C hydrophone	Sound: Grunt or moan-like sound Duration: .04 to .2 sec. Freq. span: 0 to 1.5 kc. Pred. int.: Below .5 kc.

TABLE II—*Continued*

Family	Species	Vibration frequency analysis
Diodontidae	*Diodon hystrix* hand-held in aquarium at hydrophone	Sound: Toothplate stridulation Duration: .09 sec./stridulation Freq. span: 0 to 8 kc. Pred. int.: 2.5 to 5.5 kc., with narrow intensity bands at 3, 4.3 and 5 kc.
Haemulidae	*Haemulon sciurus* hand-held in aquarium at hydrophone	Sound: Stridulation, pharyngeal teeth Duration: .02 to .1 sec./stridulation Freq. span: 0 to above 8 kc. Pred. int.: 1.5 to 4 kc.
Holocentridae	*Holocentrus ascensionis* in aquarium (Fig. 10) and at Turtle Rocks (Fig. 12); AX-58-C hydrophone	Sound: Thump-like single, or volleyed Duration: .04 to .1 sec./thump Freq. span: 0 to above 4 kc. Pred. int.: Below 1 kc.
Pomacentridae	*Pomacentrus leucostictus* in aquarium; male pursuing others of species at hydrophone	Sound: Single or repeated snaps Duration: .02 sec./snap Freq. span: 0 to 1.5 kc. Pred. int.: 0 to .8 kc.
Serranidae	*Epinephalus adscencionis* in cement tank at AX-58-C hydrophone	Sound: Vibrant grunt, single or repeated Duration: .04 to .1 sec./grunt Freq. span: 0 to .9 kc. Pred. int.: 0 to .3 kc.
	Epinephalus striatus in live car at AX-58-C hydrophone	Sound: Vibrant grunt, single or repeated Duration: .1 to .2 sec./grunt Freq. span: 0 to 2 kc. Pred. int.: 0 to .4 kc.
Tetradontidae	*Spheroides spengleri* hand-held in aquarium at hydrophone	Sound: Toothplate stridulation Duration: .07 sec./stridulation Freq. span: 0 to above 8 kc. Pred. int.: 1.3 to 3 kc.

are shown in Table I. Twelve species producing other-than-chewing sounds are listed in Table II, together with data derived from vibration frequency analysis. Of this latter group, only three species were identified as sources of sounds recorded during listening at sea—squirrelfish, Nassau grouper and black angelfish, *Pomacanthus arcuatus* (Linnaeus), all of which use the air bladder in sound production.

No individual free fishes were identified at sea as sources of stridulatory noises. Vibration frequency analysis of these latter sounds from recordings made at sea is rendered somewhat difficult by a broad band of sound with predominant intensities between 2 and 6 kc., which is characteristic of warmer seas, and which has generally been ascribed to snapping shrimp. It is not unlikely, however, that sounds created by the teeth of reef fishes (Table II—feeding of balistids) may contribute to this sound band, which on vibration frequency analysis tends to obscure upper frequency ranges of various sounds clearly discernible below the 2-kc level.

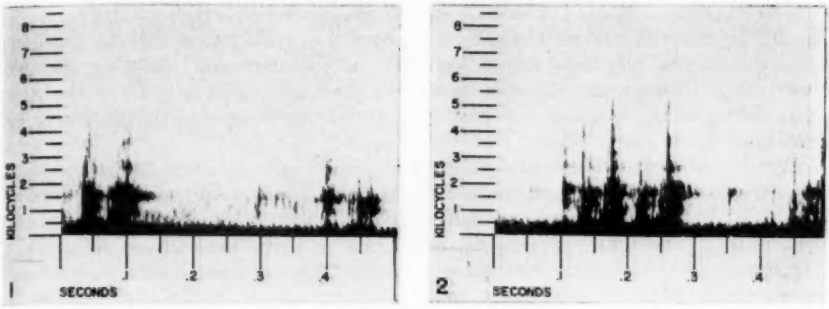


FIGURE 1. The pectoral fin-drum of *Balistes vetula* in air.
FIGURE 2. The pectoral fin-drum of *Melichthys piceus* in air.

SOUND PRODUCTION AND BEHAVIOR OF BIMINI FISHES

In the following account, each family of which sound-producing representatives were studied at Bimini, is dealt with from the points of view of the sounds produced and the correlated behavior. Initial references are to earlier descriptions dealing with sound production in the families concerned.

Balistidae (Bridge, 1910, pp. 357, 361; Fish, 1948, pp. 15–19, 1954, pp. 62–65; Schultz and Stern, 1948, p. 132). The queen triggerfish, *Balistes vetula* L., and the black triggerfish, *Melichthys piceus* (Poey), each possesses above the base of the pectoral fin a thin membrane lying lateral to the air bladder and covered by scales larger and more plate-like than those elsewhere on the body (Figs. 3, 4), a characteristic of these genera of the Balistidae (Evermann and Marsh, 1900). Males and females removed from the water and handled, frequently elevated and rapidly fluttered the pectoral fins against this region, as described by Schultz and Stern (1948), resulting in the production of a throbbing sound (Table II; Figs. 1, 2). Bridge (1910, p. 357) attributes a throbbing sound primarily to movements of the pectoral girdle.

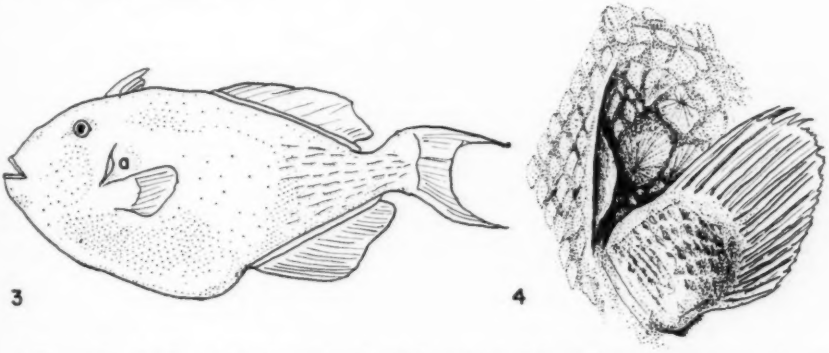


FIGURE 3. Outline of *M. piceus* showing (a) position of drumming membrane posterior to the gill opening. × 3.
FIGURE 4. Detail of the drumming membrane of *M. piceus*. × 2.5.

Differentiation of the "drumming membrane" is not apparent externally in the ocean triggerfish (*Canthidermis sabaco* Poey), nor does this species during handling move the pectoral fins to the drumming position. Similarly, the toothplate stridulation readily demonstrated by the queen and black triggerfishes (Table II; Figs. 5, 6) during handling out of water was not performed by the several ocean triggerfish studied.

While tooth stridulation and pectoral fin drumming were not heard from isolated triggerfishes handled underwater, recordings made during the feeding about the hydrophone of a captive population of queen and black triggerfishes showed predominant intensities of accompanying sounds between .6 and 2.9 kc. (Table II), which essentially spans the frequency ranges of predominant intensities obtained during tooth stridulation by these species in air. It was not possible to ascribe any specific sounds recorded at sea to triggerfishes, although their feeding activity probably contributed to background sounds recorded. They are common in the area studied.

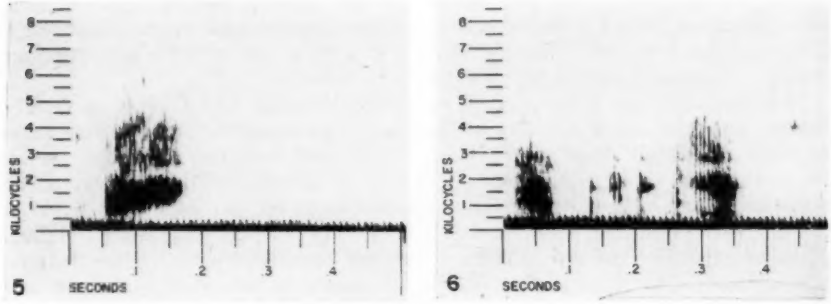


FIGURE 5. Tooth stridulation of *B. vetula* in air.
FIGURE 6. Tooth stridulation of *M. piceus* in air.

Carangidae (Bridge, 1910, p. 363; Fish, 1948, pp. 25–30). The pharyngeal tooth stridulation of *Caranx hippos* (Linnaeus) was recorded in a laboratory aquarium during handling of a 3.5-inch individual (Table II; Fig. 7). The sound recorded was not identified in any recording made at sea. Similar sound production in a related species, *Caranx crysos* (Mitchill), has been described by Fish (1954), but the thump she describes as occurring during shock was not heard during handling of the small specimen, nor was any detectable sound recorded from adult carangids swimming around and past the hydrophone at sea. A local fisherman related the stridulatory sound to "rattling of the ear bones" and asserted that a hooked specimen making this noise attracts other individuals of the species.

Chaetodontidae (Bridge, 1910, p. 361). The vibrant deep grunts of the queen, *Angelichthyes ciliaris* (Linnaeus), and black, *Pomacanthus arcuatus* (L.), angelfishes (Table II) are not easily distinguished from those of the serranids with which they may occur. Vibration frequency analysis shows (Fig. 8) a tendency for highest frequencies of the angelfish grunts to be located in the middle of the call, whereas serranid grunts tend to be initiated with high frequency spikes. Each

may vary in the direction of the other, however, and since serranids and angelfishes tend to occur in similar areas, the sounds of the two may be confused. Field and laboratory observations indicated that serranids are far more prolific in call production under ordinary circumstances than are the angelfishes.

Under laboratory conditions, both queen and black angelfishes produce the grunt during feeding on bits of conch, and when startled to quickened swimming by an observer. The deeply recessed air bladder, as seen in the black angelfish, bears no intrinsic muscles, and sound production is due to the contraction of axial musculature adjacent to the air bladder. Each quick motion of a black angelfish nibbling at the hydrophone at sea resulted in a brief grunt, although more leisurely swimming of both species in laboratory tanks was not accompanied by sound production. Handling of black angelfish under water brings forth brief grunts of low intensity, coinciding with body muscle contractions. Uniquely among species studied, the black angelfish, usually in pairs, readily approached and butted against the hydrophone at sea.

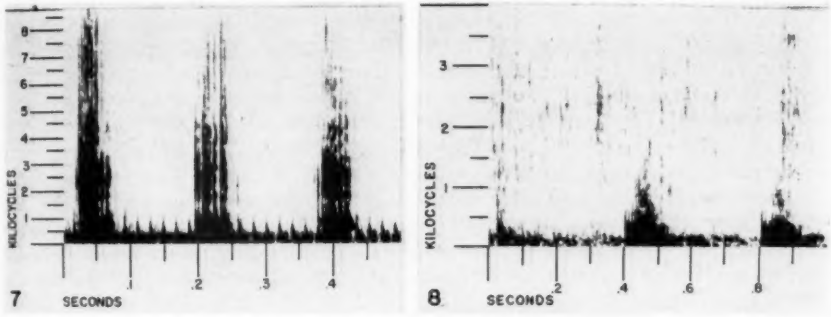
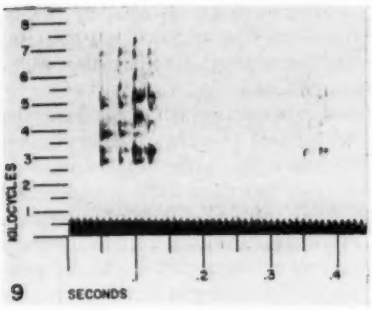


FIGURE 7. Pharyngeal tooth-stridulation of *Caranx hippos* in aquarium.
FIGURE 8. A short and two longer calls of *Pomacanthus arcuatus* west of Turtle Rocks, the latter during approach of another *P. arcuatus*. Snapping shrimp in background.

The deeply recessed position of the air bladder brings it into intimate association with surrounding peritoneum, and to the latter attach many of the axial muscle fibers heavily surrounding the slender ribs. These attached fibers appear to maintain a tension on the wall of the air bladder; cutting of the fibers creates a resonance within the bladder, and results in its partial collapse.

The maximum duration of angelfish grunts obtained during this investigation occurred west of Turtle Rocks on 10 July (Fig. 8), when an adult black angelfish examining the hydrophone and butting gently at its rubber case suddenly gave vent to prolonged, rather moan-like sounds, each of .2-sec. duration, and swam toward an approaching fish of the same species. The two fish faced each other for a few moments, after which both came quietly to the hydrophone and finally swam off together. The interpretation of a recognition signal in the prolonged grunts was rather difficult to avoid, for prior to production of the longer grunts, the first fish produced shorter, sharper sounds during its examination of the hydrophone. On another occasion, 12 July in the same area, sounds similar to the shorter grunts



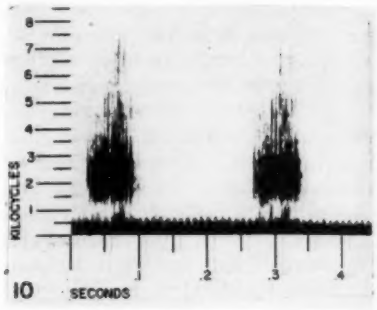
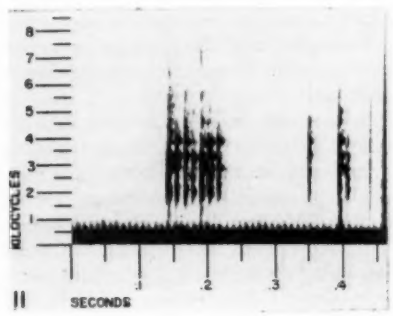


FIGURE 9. Toothplate stridulation of *Diodon hystrix* in aquarium.
FIGURE 10. Toothplate stridulation of *Spheroides spengleri* in aquarium.

were recorded as a pair of black angelfishes butted several times against a pair of cowfish, *Lactophrys tricornis* (L.), approximately 6 feet from the hydrophone.

No sounds were recorded from the single species of butterflyfish studied, *Chaetodon striatus* L., nor were sounds recorded from an immature specimen (4-inch, total length) of the French angelfish, *Pomacanthus paru* (Bloch).

Diodontidae, Tetradontidae (Fish, 1948, 1954; Burkenroad, 1931). The porcupinefish, *Diodon hystrix* L., and the puffer, *Spheroides spengleri* (Bloch), were so similar in acoustical behavior as to merit a single discussion (Table II). Both produce sound during and after inflation by stridulation of the toothplates, the sound being of a klaxon-like variety (Figs. 9, 10). Only feeding sounds were recorded from undisturbed individuals. Frequencies of greatest intensities are similar in chewing and stridulation noises, although these levels vary between the two species (Table II) and may be expected to vary with size. Only a single individual of each was recorded at Bimini. Observations on these species and on the common puffer, *Spheroides maculatus* (Bloch and Schneider) of the Woods Hole area suggest that the stridulatory sound is more readily elicited from smaller individuals than from full-grown animals.



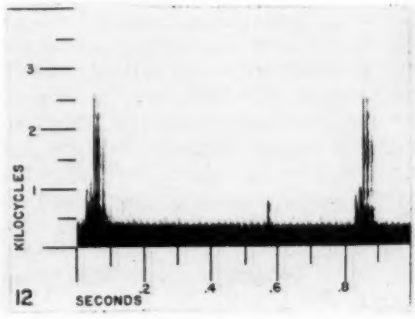


FIGURE 11. Pharyngeal tooth stridulation of *Haemulon sciurus* in aquarium.
FIGURE 12. Single thump-like sounds of *Holocentrus ascensionis* in cement tank.

Haemulidae (Burkenroad, 1930, 1931; Fish, 1948, pp. 63–66; Schultz and Stern, 1948, p. 131). Although the fishes called grunts are notoriously noisy species of warmer marine waters, due to the rasping stridulation of the pharyngeal teeth, observations at Bimini indicated that not all species of the Haemulidae are equally important sound producers (Tables I and II). The haemulid rasp was not identified in any recordings made at sea; it was heard only from hand-held specimens of the blue-striped (Table II; Fig. 11) and yellow grunts, *Haemulon sciurus* (Shaw) and *H. flavolineatum* (Desmarest), respectively; no recordings

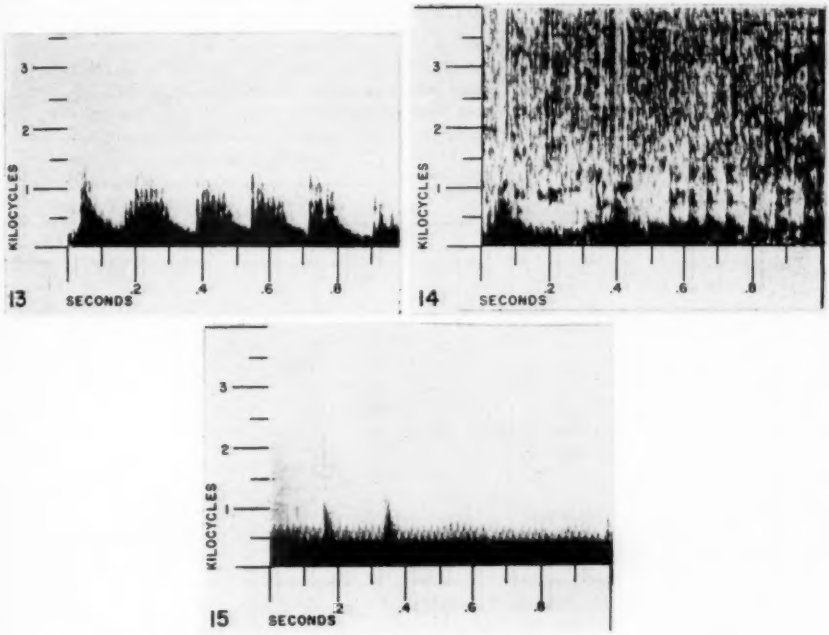


FIGURE 13. Grunts of *Epinephalus striatus* in cement tank.
FIGURE 14. Two *E. striatus* grunts (left) and a volley of thump-like sounds of *H. ascensionis* west of Turtle Rocks. Snapping shrimp in background.
FIGURE 15. Snaps of male *Pomacentrus leucostictus* in aquarium.

of the latter were obtained. The observations of Burkenroad (1930) which related the sounds of *Haemulon* to movements of the pharyngeal teeth were confirmed, with the exception that the association between the dorsal pharyngeal teeth and the anterior end of the air bladder seems more important in resonating the rasping sound than does the varying relation between the air bladder and the lower pharyngeal teeth. The opercula are somewhat extended during sound production. Rubbing together of dissected pharyngeal toothplates produces a much fainter sound than that produced by the living fish.

Holocentridae (Fish, 1948). Sound production by the squirrelfish, *Holo-*

centrus ascensionis, said to have derived its name from its chattering call (D. de Sylva, personal communication), is characteristic of the western edge of the Great Bahama Bank (Table II; Fig. 12, 14). It was recorded only along, and mainly to the west of, the island and rock chain extending from North Bimini south to South Cat Cay (Tables III and IV; Fig. 16). In rocky areas of the bottom along a relatively narrow area probably extending not far below the limits of visibility from the surface, squirrelfish are characteristically found in the daytime each hovering near a depression approximately large enough to receive a single fish. Generally living in higher parts of ledges than the grouper, an occasional squirrelfish inhabits a rocky fissure with *E. striatus.* Collection of squirrelfish in traps up to

TABLE III

Grouper and squirrelfish sound production along the western edge of the Great Bahama Bank in the Bimini area

Recording station	Duration of recording	Calls/minute		
		Grouper	Squirrelfish	Both
1) At 15 fa. depth W of Moselle Shoal, drifting N	13 min.	.08	0	.08
2) At 25 fa. depth SW of (1), drifting N	18 min.	.4	0	.4
3) W of gap between Round and Turtle Rocks	6 min.	1.0	5.2	6.2
4) W of Turtle Rocks	5 min.	.2	7.2	7.4
5) W of Turtle Rocks	11.5 min.	.8	6.3	7.1
6) W of Turtle Rocks	45 min.	1.9	9.5	11.4
7) W of Turtle Rocks, drifting SE	14 min.	.6	3.4	4.0
8) W of Triangle Rocks, drifting NE	10 min.	1.3	3.3	4.6
9) West of Triangle Rocks	13 min.	1.7	3.2	4.9
10) W of gap between Piquet and Triangle Rocks	15 min.	2.5	8.6	11.1
11) W of Piquet Rocks	12 min.	2.4	4.1	6.5

Average number of calls/minute (11 stations):

Grouper	1.2
Squirrelfish	4.6
Both	5.8

a mile east of Turtle Rocks and occasional sightings in deeper reaches east of the Bimini-Cat Cay chain indicated that the species may move beyond its more common daytime habitat along the outer face of the Bank. Holocentrids are mainly nocturnal in their habits (Barbour, 1905, p.119; Randall, 1955, pp. 33, 38) and at night their local distribution may be considerably more dispersed than during the daytime, although the species considered here is said to return to the same hole each day (Ray and Ciampi, 1956, p. 207).

Squirrelfish sounds are produced by contractions of body wall musculature against the rather firm-walled air bladder which is closely associated with the rib cage. The first three ribs are expanded, flattened and thinned dorsally, and are intimately associated with the air bladder wall; more posterior ribs are easily

separated from the bladder. Physiological stimulation indicated that the musculature chiefly responsible for sound production was that attaching to the dorsal portions of the first three ribs, which appear to serve as drumheads. The structure of the air bladder, and its close relationship with the auditory region of the skull, have been described by Nelson (1955).

The thump-like sounds of squirrelfish may be repeated singly at irregular intervals or produced in rapid volleys; they are more sharply peaked on vibration frequency analysis than are the sounds of angelfish and grouper. When produced by

TABLE IV

Grouper and squirrelfish sound production over the Great Bahama Bank in the Bimini Area

Recording station	Duration of recording	Calls/minute		
		Grouper	Squirrelfish	Both
1) On Moselle Shoal	23 min.	.04	.06	.1
2) Over wreck ENE of North Rock	27 min.	.4	0	.4
3) At North Rock, E side	27.5 min.	.04	.2	.2
4) At Crossing Rocks, W side	37.5 min.	.1	1.0	1.1
5) In Bimini Harbor entrance, drift to N	6.5 min.	0	.6	.6
6) 50′ off S. Bimini, W side	13 min.	.7	.2	.9
7) At Round Rock, W side	8 min.	1.0	0	1.0
8) At Round Rock, E side	9 min.	.3	0	.3
9) On line between Turtle and Round Rocks, drift NW	10 min.	.9	1.9	2.8
10) Same station	30 min.	1.4	1.8	3.2
11) North end of Turtle Rocks, E side	4 min.	0	0	0
12) Same station	3 min.	0	0	0
13) On line between Triangle and Turtle Rocks, drift NE	12 min.	.4	2.2	2.6
14) Concrete shipwreck, E of Turtle Rocks	9 min.	0	0	0
15) Triangle Rocks, E side	11 min.	0	.6	.6
16) Dollar Harbor	5.5 min.	0	0	0
17) Wedge Rocks, NE side	8 min.	.8	1.4	2.2

Average number of calls/minute (17 stations):

Grouper	.3
Squirrelfish	.5
Both	.8

startled or handled squirrelfish, the sounds are volleyed in bursts of 4 to 20. Singly-produced sounds are of longer duration than those produced in volleys; these singly-produced sounds are not dissimilar to the ear from the sounds of Nassau grouper, although they cover a greater frequency span than do those of the grouper when both are recorded at the hydrophone.

Undisturbed squirrelfish confined in laboratory aquaria and outdoor tanks remained silent during listening periods of up to three hours; confined specimens produced sounds only when handled, fed or startled. Quickened swimming during feeding was characterized by sound production of a considerably lower intensity

than that produced during handling of the fish. Sudden startling of confined squirrelfish generally resulted in volleys of sound production.

At sea, squirrelfish were sometimes heard when no individuals were sighted, but the acoustical behavior of the species was characteristic when specimens were approached by the suspended hydrophone at drift stations. This behavior included

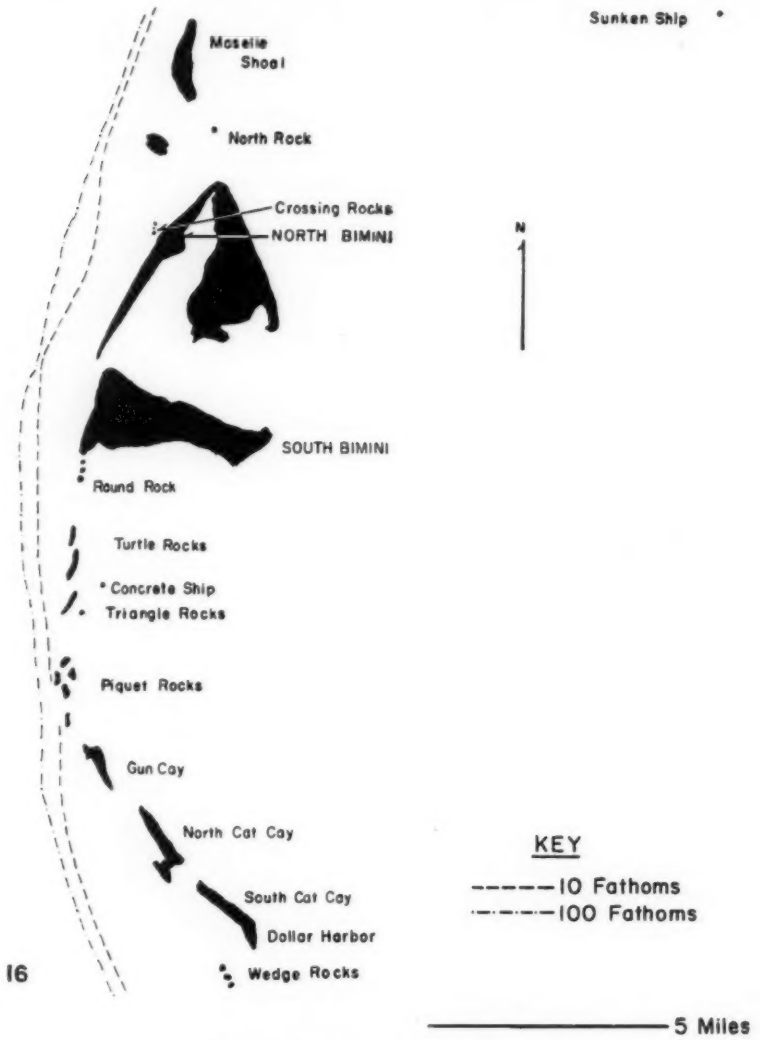


FIGURE 16. The Bimini area from Moselle Shoal to South Cat Cay.

erection of the spiny fins, adjustment of position so that an eye was directed toward the approaching hydrophone, movement toward a rocky depression near which the fish hovered, and production of the call in volleys of 3 to 20 individual pulses. The continuing approach of the hydrophone resulted in the fish's moving into its rocky shelter.

Pomacentridae (Fish, 1948, pp. 59–63). The sharp but rather faint knocks or snaps produced probably only by males of the demoiselle, *Pomacentrus leucostictus* (Mueller and Troschel), were recorded when a given individual suddenly dashed from cover to pursue other individuals approaching its place of concealment (Table II; Fig. 15). These were heard on several occasions. The behavior was similar to that of a sciaenid, *Corvina nigra* (Bloch) described by Dijkgraaf from Naples (1947).

Serranidae (Fish, 1954, pp. 36–44). The air bladder of the Nassau grouper, *Epinephalus striatus*, is thin-walled and lacks intrinsic muscles, yet this species equals the squirrelfish in its importance as a source of marine sound in the Bimini area (Table II; Figs. 13, 14). Contractions of body wall musculature appear to be responsible for the sound production, while the unusually heavy peritoneum stretched over the air bladder and adjacent organs apparently acts as the sounding board. Appropriate stimulation of opened fishes brought about some sound production, even after deflation of the air bladder. The strong contractions of body wall musculature accompanying production of the call are visible externally, and are easily elicited by startling or handling the fish in laboratory tanks. Sounds similar to those produced on alarm, but of a somewhat lower intensity, are produced during quickened swimming accompanying feeding.

Like the squirrelfish, the Nassau grouper inhabits the underwater ledges west of the Bimini-Cat Cay chain of rocks and island (Fig. 16). Its distribution is broader from east to west than that of the squirrelfish, since it was sighted where rocky fissures occurred in an otherwise sandy bottom in the Bimini lagoon, and it was seen and recorded in a shipwreck approximately 11 miles northeast of North Bimini Island on the Great Bahama Bank (Station 2, Table IV; Fig. 16). Unidentified sounds thought to originate from serranids were recorded at depths exceeding 15 fathoms west of North Bimini Island (Stations 1 and 2, Table III), from which depths serranids have been obtained at Bimini (Scholander *et al.*, 1951; Scholander and van Dam, 1954).

The Nassau grouper tends to be more secretive in its habits than the squirrelfish, ordinarily lying deeper in rocky fissures or well beneath overhanging ledges on the outer faces of which squirrelfish are more frequently seen. In acoustical behavior, the two are similar. As the suspended hydrophone approaches a grouper, the fins are erected and the fish retreats to cover, accompanying the retreat with the characteristic vibrant grunts. Like the call of the squirrelfish, that of the grouper was sometimes heard when no individual was sighted. The call is a characteristic marine sound of the area immediately west of the Bimini-Cat Cay chain.

The sound of the rock hind, *Epinephalus adsencionis* (Osbeck) (Table II), while of markedly lower intensity than that of the Nassau grouper, is produced under similar circumstances in laboratory tanks and is of similar characteristics. It was never specifically recognized during recording at sea. No sounds whatever were recorded from a captive *Promicrops itaiara* (Lichtenstein) weighing in the

neighborhood of 300 pounds, although the fish on one occasion very nearly swallowed the hydrophone.

Distribution of Grouper and Squirrelfish Sounds in the Bimini Area

The distribution of squirrelfish and Nassau grouper in the Bimini area has been summarized above. Determination of this distribution was based on both visual and aural observations. The sounds of these species were selected to explore the possibility of determining the distribution of sonic species by aural means alone.

The total area of observation extended approximately 20 miles along the northwest edge of the Great Bahama Bank (Fig. 16), from Moselle Shoal on the north to Wedge Rocks on the south, and in an east-west direction from the location of a sunken freighter on the Great Bahama Bank (25° 49′ N, 79° 7′ W) 10.7 miles on a heading of 75 degrees from the northern tip of the Bimini Islands, to a station over an approximate depth of 150 feet southwest of Moselle Shoal. Recordings were made at 28 stations (Tables III and IV), all but two (Stations 1 and 2, Table III) being approximately on or within the 6-fathom line to permit observation of fishes during recording. Table III includes those stations, fixed and drift, at which recordings began on or were wholly confined to the edge of the Great Bahama Bank, to the west of the Bimini-Cat Cay chain; Table IV includes those stations located further in upon the Bank, in most cases immediately to the east of the Bimini-Cat Cay chain. The stations are in each case listed in order from north to south, and are referable to Chart No. 1854 of the United States Hydrographic Office, and to U. S. Coast and Geodetic Survey Chart No. 1112.

While not each call ascribed to either grouper or squirrelfish in Tables III and IV has been analyzed by vibration frequency analysis, a broad sample of recordings made at sea has been thus treated, and the results have indicated that the interpretations of recordings upon which Tables III and IV are based are correct. In erecting Tables III and IV, a rapid volley of either grouper or squirrelfish sounds has been characterized as a single call. Closely consecutive calls of the same species but of different individuals are usually interpretable as such on the basis of intensity differences, due to the origins' being at different distances from the hydrophone.

It will be noted in Table III that, although squirrelfish calls were lacking in the deepest stations recorded (1 and 2), along the sloping edge of the Bank they predominated over grouper calls by a factor of nearly 4. Moving of the listening station from the slope side of the Bimini-Cat Cay chain immediately to the Bank side (Table IV) resulted in an abrupt drop in incidence of calling by both species, but particularly by the squirrelfish. On the Bank itself, where squirrelfish were much less frequently sighted, their calls predominated over grouper calls by a factor of less than 2. The calling of these two species together was over 7 times as frequent along the edge of the Bank as to the east of its edge (Tables III and IV). The difference in incidence of calling between slope and Bank sides of the Bimini-Cat Cay chain is exemplified especially by comparison of Table III, Stations 4, 5 and 6, with Table IV, Stations 9, 10, 11 and 12. Short moves of the listening station resulted in marked differences in the incidence of underwater biological sounds.

Although not indicated by the data presented in Tables III and IV, observations at drift stations (Table III, Stations 7 and 8; Table IV, Stations 9 and 13) indicated that as the hydrophone moved over alternately sandy and rocky bottom, the incidence of calling rose markedly as the boat moved over underwater ledges and fell over the sandy stretches; the incidence of calling thus provided an indication as to the type of bottom beneath the boat, and correlated with sightings of grouper and squirrelfish.

Other Underwater Sounds of the Bimini Area

Sounds most frequently heard during the underwater listening in the Bimini area, other than those described, are (1) the snapping and crackling characteristic of tropical seas, generally attributed to snapping shrimp, but actually indistinguishable by methods commonly employed from the sounds of some stomatopods (Johnson, *et al.*, 1947; Moulton, 1957); (2) a rattling sound like that produced by the spiny lobster, *Panulirus argus* (Moulton, 1957); and (3) another unidentified rattling sound with its predominant frequencies lying between .5 and 1.3 kc., each pulse being of .02 second duration, and repeated at intervals of approximately .12 second in volleys of varying length. In addition to these, a distinctive series of sounds was recorded twice the same day (12 July 1956 at Stations 6 and 9, Table IV). At Station 6, the sound was a buzz-like whine singly produced; at Station 9, the same sound was preceded by a number of sharp metallic raps and was followed by a number of brief chirps of somewhat lower frequency than the raps. The metallic raps were so similar to the sound of pounding on a steel hull that two observers, prone at the glass panels, concluded a vessel to be bearing down on the listening post. Since the listening boat was quiet except for water noise along the hull, and since no other boats were within view to the horizon, it is assumed that the sounds were of biological origin, but the source is unknown. They do not correspond with known cetacean sounds (Mr. William Schevill, personal communication).

Discussion

The behavior of the Nassau grouper and squirrelfish in the Bimini area furnishes a marked exception to the generalization (Fish, 1954, p. 7) that fishes in the field are silenced by strange contacts. Both of these species were obviously stimulated to active sound production by approach of the boat and suspended hydrophone at drift stations over shallow waters. The spiny fin erection and movement toward concealment of these species upon approach of the hydrophone were strongly suggestive that the sounds described are related to self-protection, a probability further suggested by production of the same sounds during handling of these fishes. Although some fishes may use echo-location (Griffin, 1955), there is no evidence at present of its being involved in the cases under discussion. Circumstances surrounding production of grouper and squirrelfish calls, as observed at Bimini, were similar to those that surround production of sea robin grunts (Moulton, 1956b). While the grunts are readily produced during handling by most specimens of the sea robins common at Woods Hole, *Prionotus evolans* (L.) and *P. carolinus* (L.), the grunts are also produced by sea robins contained together in live cars and living on the bottom. Sea robin grunts are not, however,

so easily stimulated by startling as are the sounds of Nassau grouper and squirrelfish which readily produced sounds recorded at sea after periods of confinement of two weeks in laboratory aquaria.

The squirrelfish is the most significant producer of underwater sound among fishes in the Bimini area, and if the 20-mile extent of the Bank area studied may be considered typical of the whole, of the edges of the Great Bahama Bank generally. In view of this significance, Fish's (1948, p. 44) estimate of the sonic importance of the Holocentridae as "probably none" must be rejected. Since holocentrids are of wide distribution in tropical and sub-tropical waters, it seems probable that their sonic importance extends to other waters than those of the Bimini area.

The acoustical behavior of the angelfishes (Chaetodontidae) has not been hitherto adequately described, but there can be little doubt that the behavior of the single specimen recorded at Turtle Rocks on 10 July furnished evidence of a call accompanying recognition behavior in this species. The black angelfish which is most common of the angelfishes in the Bimini area, has a tendency to examine underwater objects (hydrophone, swimming cowfishes), such examination being accompanied by sounds of briefer duration than those proposed as a part of recognition behavior. The black angelfish is usually observed in pairs during July and August at Bimini. It seems likely that chaetodontids may contribute to underwater sound in other tropical and sub-tropical areas, although they are not discussed among sound-producing fishes of the Pacific by Fish (1948).

Further evidence of the tendency of captive fishes to become silent unless disturbed was provided by all species studied during the summer of 1956. The only species to produce sound spontaneously in laboratory aquaria during listening periods of up to three hours, other than those produced during feeding and being startled, was the small pomacentrid, *Pomacentrus leucostictus*, probably a male, as it pursued other individuals encroaching on its hiding place.

As is the case along the northeastern coast of the United States where the most significant fish producers of underwater sound which have been identified (sciaenids, triglids, and batrachoidids) are fishes using, rather than skeletal stridulatory mechanisms, muscles in close association with the air bladder (Tower, 1908; Fish, 1954; Moulton, 1956b), a holocentrid, a serranid and a chaetodontid are the most frequently calling fishes of the area immediately about the Bimini-Cat Cay chain. Of the former, however, both sea robins and toadfish produce sounds with muscles intrinsic to the air bladder, while all three of the most important calling fishes of the Bimini area use muscles extrinsic to the air bladder in producing their sounds.

The structural specializations and behavioral patterns of the sound producing species studied at Bimini have provided further striking evidence of the significance of sound in the biology of the species concerned, and the consistent incorporation of sound production into behavioral patterns observed in the clear water about Bimini (Nassau grouper, squirrelfish, angelfish) strengthens a conclusion that sound is of significance to the species concerned. Yet, as has repeatedly been affirmed, clear evidence of effectiveness of the sounds concerned in modifying the movements of fishes in nature is still lacking, although Tavolga (1956) has observed the females of a goby to demonstrate increased activity during sound production

by the male in breeding. The sounds of all calling species studied at Bimini are produced by both sexes, except for the snaps of *Pomacentrus leucostictus*.

Experiments of playing into the water artificial sounds and recordings of natural sounds such as those that have elsewhere modified fish behavior (Moulton, 1956a, 1956b; Tavolga, 1956) had no notable effect in the Bimini area. During all listening at sea in the Bimini area, all during daylight, sound production was prolific or rare, depending on the distribution of the species concerned and not on alternating periods of quiet and of sound production which seem to characterize production of the staccato call of sea robins at Woods Hole (Moulton, 1956b). Therefore, these experiments are not reported in detail. The Nassau grouper and squirrelfish were never observed in areas where their calls were not heard at Bimini.

The most characteristic component of background noise in the Bimini area is the "crackle" so characteristic of warmer seas, and which has generally been ascribed to snapping shrimp (Johnson *et al.*, 1947). By the analysis methods used, this noise in the Bimini area presents components cumulatively spanning the frequency range examined (up to 8 kc.). The invertebrates largely responsible are a common stomatopod (*Gonodactylus oerstedi*) and several kinds of snapping shrimps, including *Alpheus armatus* and *Synalpheus* spp. (Johnson *et al.*, 1947; Pearse, 1950; Moulton, 1957). The sound spectra obtained during this study from recordings of feeding and of stridulation sounds of various fishes indicate that in regions where producers of such sounds are numerous, they may contribute extensively to the spectrum of underwater sounds generally attributed to invertebrate sound producers.

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Summary

1. On the basis of observations and recordings at sea and in the laboratory, the acoustical behavior of 13 species of Bahamian fishes is described, and their sounds are defined. Twenty-six species producing no calls in the course of this study are specified.

2. The most important sound-producers among fishes of the Bimini area are the squirrelfish, *Holocentrus ascensionis*, and the Nassau grouper, *Epinephalus striatus*. Their characteristic sounds may be anticipated when these species encounter a strange object at sea, and probably generally during the daytime along the edges of the Great Bahama Bank.

3. A single observation has indicated that calling is a component of recognition behavior in the black angelfish, *Pomacanthus arcuatus*. The families Chaetodontidae and Holocentridae should be added to lists of fish families containing calling members.

4. The usefulness of underwater listening in studying the distribution of some calling fishes has been demonstrated in the cases of the squirrelfish and Nassau grouper.

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EFFECT OF PLANT HORMONES ON ULVA[1]

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Föyn (1934a) in his early attempts to grow *Ulva lactuca* found that *Ulva*, like *Cladophora suhriana* (Föyn, 1934b), grows poorly in sea water enriched with nitrates and phosphates (Schreiber, 1927) and that the addition of soil extract to Schreiber's medium is necessary to obtain normal growth and the entire life-cycle. This medium ("Erdschreiber") later became the standard medium for growing marine flagellates in bacterized cultures (Gross, 1937; Parke, 1949).

Kylin (1941) employed *Ulva lactuca* to analyze the biological activity of different samples of sea water: he found that sea water at 70 meters depth is inadequate to support normal growth and that addition of nitrates, phosphates and trace metals made it suitable for the germination of the zoospores of *Ulva* and elicited as rapid growth of the germlings to the stage of 15–20 cells as did the "Erdschreiber."

Levring (1946), employing the same technique and test organism, formulated a synthetic sea water which, similarly enriched, allowed normal development of the germlings of *Ulva*, thus confirming, with a chemically defined medium, Kylin's conclusion. Levring's medium was the starting point for the formulation of several synthetic marine media which do not precipitate and are suitable for the cultivation of a number of marine and brackish algal flagellates in bacteria-free culture (Provasoli, McLaughlin and Droop, 1957).

Since Föyn, Kylin and Levring worked with bacterized cultures, I wondered if *Ulva*, when bacteria-free, would grow in mineral media or if it would require organic factors. Many other algae which, like *Ulva*, were previously cultured in Erdschreiber + bacteria, require, besides nitrates and phosphates, growth factors and trace metals when cultured in synthetic media without bacteria (Provasoli and Pintner, 1953; Sweeney, 1954; Lewin, 1954; Droop, 1955a, 1955b, 1957; Provasoli, 1957).

In exploratory attempts to grow *Ulva* in bacteria-free culture, I failed to obtain a typical foliaceous thallus but in trying to obtain it, I found that *Ulva* germlings respond to plant hormones.

Material and Methods

Bacteria-free cultures of *Ulva* were obtained by placing pieces of thallus on the surface of agar media containing various concentrations of an antibiotic mixture (1 ml. of the concentrated antibiotic solution contains: K penicillin G 12,000 units; chloramphenicol 50 μg.; polymyxin B 50 μg.; neomycin 60 μg.).

I recognized from the beginning the necessity of employing thalli free from epiphytic organisms: the pieces of thallus were selected and inspected under the dissecting microscope and, as an additional precaution, were cleaned by brushing

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the two surfaces with a thick, soft water-color brush. Even so, the epiphytes could not always be eliminated and several bacteria-free cultures of *Ulva* were infected with small diatoms (mainly *Nitzschia*).

The concentrated antibiotic mixture was sterilized by filtration through a glass filter, and 0.1-, 0.2-, 0.3-ml. aliquots were dispensed on the bottom of sterile Petri dishes; 20 ml. of sterile 1.4% agar media, kept at 45° C., were added and thoroughly mixed with the antibiotic by twirling. The pieces of *Ulva* thallus (5-mm. squares) were left on this agar for 7 days, removed aseptically with a spatula, placed in depression slides containing sterile media, cut in several narrow strips with an iridectomy scalpel, and transferred to liquid media. The pieces treated with the lower concentrations of antibiotics (0.1–0.2 ml. in 20 ml. of agar media) were infected with either bacteria, a pink yeast, or diatoms. All the pieces treated with 0.3 ml. of antibiotic mixture (final concentration of antibiotics per ml. of agar medium: K penicillin 200 units, and 1 μg. each of chloramphenicol, neomycin, and polymyxin) were bacteria-free but about ⅓ were infected by diatoms.

Several liquid media were tried: the most successful were ASW III and ASW 8 (Table I); both are enriched sea water media similar to, but richer than, Erdschreiber. ASW III (richer in organics) was employed in the early experiments; later I employed ASW 8 which allows better growth.

The cultures are carried in screw-cap tubes (125 × 20 mm.) with 10 ml. of medium; to avoid chemical contamination we employ plastic caps without liners. At first, *Ulva* was grown in continuous light (200 foot-candles; fluorescent tubes) at 18–20° C., later in alternate light (16 hours) and darkness (8 hours).

The sample of gibberellins was kindly supplied by Dr. Nickell, of Chas. Pfizer & Co., and the one of kinetin bought from the California Foundation for Biochemical Research.

Results

The purified strips of thallus, when transferred to liquid media, began to produce thin filamentous germlings from their surface and looked like pincushions. That many zoospores were also set free was evident from the many small germlings that covered the walls of the test tubes.

The germlings arising from the thallus were round, thin, solid, and never became more than 2–4 mm. long; after two months they bleached, leaving a few intensely green spots which dotted their surface at random. The germlings on the walls of the tubes in certain media behaved similarly and produced a number of rhizoids, many of them colorless; in other media, the germlings developed only rhizoids and looked like stellate colonies or like an elongated root system in miniature. Pieces of bleached stubby germlings, or the stellate rhizoidal colonies, when transferred to new media, produce new germlings from the islands of intensely green cells which are scattered among the bleached tissues; these green cells remain dormant and viable for a year (longest time tried) in the old medium.

The germlings of the second generation underwent a similar cycle, they grew a few millimeters and later bleached partially; no zoospores were produced by these germlings. Serial transfers are carried out by removing the old germlings aseptically from the culture tubes, cutting them in pieces and inoculating the pieces in different media.

Föyn obtained normal development of the thallus, production of zoospores and gametes of *Ulva*, in bacterized cultures grown in Erdschreiber. ASW III, an Erdschreiber enriched with vitamins, liver extract, and carbon sources, allowed only the formation of germlings; the typical thallus was never obtained in bacteria-free cultures in this medium. The beginning of a thallus (the formation of two short, thick "rabbit ears" or a fan-like curly, thick, small thallus) was obtained in

TABLE I

Media for Ulva

	Föyn's Erdschreiber	ASW III	ASW 8
Sea water	100 ml.	100 ml.	80 ml.
H_2O			20 ml.
$NaNO_3$	10 mg.		30 mg.
KNO_3		20 mg.	
$Na_2HPO_4 \cdot 12\ H_2O$	2 mg.		
K_2HPO_4		2 mg.	
Na_2 glycerophosphate			3 mg.
Mn (as Cl)		0.04 mg.	
Fe (as Cl)		0.01 mg.	0.05 mg.
P II metals*			3 ml.
Vitamin mix No. 8**		0.1 ml.	
Vitamin mix S. 3***			0.5 ml.
B_{12}			0.01 μg.
Liver 1:20†		1. mg.	
Soil extract	5 ml.	4 ml.	
Na H glutamate		50 mg.	
Glycine		50 mg.	
Tris (hydroxymethyl) amino-methane††		100 mg.	100 mg.
pH	8.0	7.5	8.0

* One ml. of P II metal contains: ethylenediamine tetraacetic acid, 1 mg.; Fe (as Cl) 0.01 mg.; B (as H_3BO_3) 0.2 mg.; Mn (as Cl) 0.04 mg.; Zn (as Cl) 0.005 mg.; Co (as Cl) 0.001 mg.

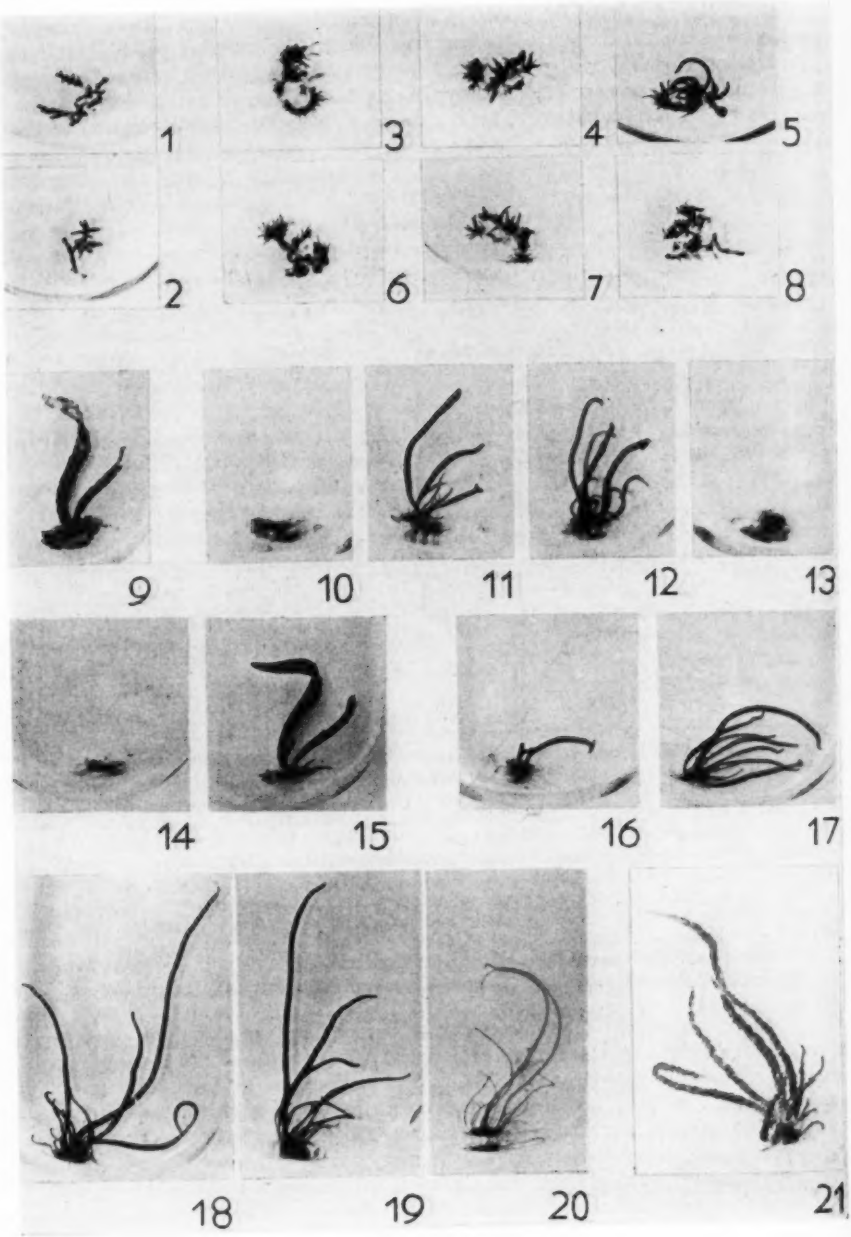
** One ml. of Vitamin mix No. 8 contains: thiamine HCl, 0.2 mg.; nicotinic acid, 0.1 mg.; putrescine 2 HCl, 0.04 mg.; Ca pantothenate, 0.1 mg.; riboflavin, 5.0 μg.; pyridoxine 2 HCl, 0.04 mg.; pyridoxamine 2 HCl, 0.02 mg.; para-aminobenzoic acid, 0.01 mg.; biotin, 0.5 μg.; choline H_2 citrate, 0.5 mg.; inositol, 1.0 mg.; thymine, 0.8 mg.; orotic acid, 0.26 mg.; B_{12}, 0.05 μg.; folinic acid, 0.2 μg.; folic acid, 2.5 μg.

*** One ml. of Vitamin mix S. 3 contains: thiamine HCl, 0.05 mg.; nicotinic acid, 0.01 mg.; Ca pantothenate, 0.01 mg.; *p*.-aminobenzoic acid, 1.0 μg.; biotin, 0.1 μg.; inositol, 0.5 mg.; folic acid 0.2 μg.; thymine, 0.3 mg.

† Nutritional Biochemical Corporation, Cleveland, Ohio, U. S. A.

†† "Sigma 7–9 biochemical buffer," Sigma Chemical Co., St. Louis, Missouri, U. S. A.

two of the tubes which were infected with a pink yeast or a diatom. However, re-infection of the axenic cultures of *Ulva* germlings with clonal cultures of the yeast or diatom failed to produce a thallus. Disappointed by the failure to obtain normal thalli, I re-examined Föyn's cultural methods and noted that he had found (1955) that only the northern European variety of *Ulva* can be grown in continuous light, that the southern variety (which he proposed to call *Ulva thureti*) bleached if grown in continuous light and that a normal thallus was formed only with a daily period of 6 or more hours of darkness. From then on, all cultures had an 8-hour



FIGURES 1–21.

dark period and 16 hours light but no thallus formed and the germlings bleached as before.

The repeated failure to obtain normal morphogenesis of the thallus in bacteria-free culture, in media similar to Erdschreiber, suggested that the failure was due to a lack of morphogenetic regulators; some of them, like indolacetic acid and gibberellin, can be produced by microorganisms. I tried various concentrations and combinations of adenine, indolacetic acid (IAA) and kinetin which influence growth and differentiation in higher plants.

In the first experiments we found that adenine by itself favored the production of more germlings from the dormant green cells and induced longer filaments; the best concentration was 3 mg.% (6 mg.% is inhibitory).

In the presence of 1 μg.% kinetin, indolacetic acid induced a large number of germlings whose length tended to increase proportionally with the concentration of IAA: 5 μg.% was the best. When 3 mg.% adenine was superadded, the most effective concentration of IAA was 10μg.%; 30μg.% inhibited the length and number of germlings.

In the presence of 5μg. IAA, increasing concentrations of kinetin also favor the elongation of germlings; the highest concentration tried (10 μg.%) induced the longest germlings obtained in these early experiments (Fig. 5). When adenine was superadded, the number of germlings induced by the combined action of IAA and kinetin is apparently not affected, but adenine completely inhibits the sharp elongation produced by 10 μg.% kinetin (Fig. 8). Perhaps in *Ulva* these morphogenetic determinants interact and have a limited specific action paralleling their activities on the tissues of higher plants.

At this point, we tried to substitute sea water media with synthetic mineral

EXPLANATION OF PLATE I

FIGURES 1–8. Medium ASW III: forty-five days' growth. The new growth is represented by the lateral filaments budding from the old pieces. FIGURE 1: ASW III alone. FIGURE 2: + kinetin 2.5 μg.%.

FIGURES 3–5. Kinetin curve: basal medium = ASW III + indolacetic acid 5 μg.%; FIGURE 3: + kinetin 1 μg.%; FIGURE 4: + kinetin 5 μg.%; FIGURE 5: + kinetin 10 μg.%.

FIGURES 6–8. Kinetin curve: basal medium = ASW III + indolacetic acid 5 μg.% + adenine 3 mg.%; FIGURE 6: + kinetin 1 μg.%; FIGURE 7: + kinetin 5 μg.%; FIGURE 8: + kinetin 10 μg.%.

FIGURE 9. ASW 8 medium + adenine 3 mg.% + kinetin 20 μg.%. Same as Figure 15 but after 120 days' growth. Note islands of green, resistant cells interspersed in the bleached tissue of the blade.

FIGURES 10–20. ASW 8 medium: sixty days' growth. FIGURES 10–13. Indolacetic acid curve: basal medium = ASW 8 + kinetin 10 μg.%; FIGURE 10: No addition; FIGURE 11: + indolacetic acid 5 μg.%; FIGURE 12: + indolacetic acid 10 μg.%; FIGURE 13: + indolactic acid 20 μg.%.

FIGURES 14–15. ASW 8 medium + adenine 3 mg.%; FIGURE 14: No addition; FIGURE 15: + kinetin 20 μg.%.

FIGURES 16–20. Gibberellins curve: basal medium = ASW 8 + indolacetic acid 5 μg.% + kinetin 10 μg.%. FIGURE 16: No addition; FIGURE 17: + gibberellins 1 μg.%; FIGURE 18: + gibberellins 10 μg.%; FIGURE 19: + gibberellins 40 μg.%; FIGURE 20: + gibberellins 100 μg.%: note that all the filaments are bleached and that only the rhizoids of the disc of attachment are still green.

FIGURE 21. Same as Figure 18, but after 120 days' growth; note the many knobby islands of resistant green cells interspersed on the bleached filaments.

Enlargement of all figures 2 × natural size.

media (Provasoli *et el.*, 1957) or with other types of enriched sea water to which we added the most effective hormone combination (*i.e.*, IAA 5 μg.% and kinetin 10 μg.%). ASW 8 was far better than both ASW III and the synthetic media, and was used from then on.

In ASW 8, formation of germlings and germling elongation was again favored by the combination of kinetin and IAA. With kinetin constant at 10 μg.%, only rhizoids were formed when IAA was absent; 10 μg.% IAA elicited longer germlings than 5 μg.% IAA after 30 days growth, but at this concentration the tips of the germlings became brown in 60 days and the germlings were completely brown in 90 days (Fig. 12). The combination of kinetin 10 μg.% and IAA 5 μg.% produced healthy green germlings which kept on growing and the tips began to flatten, as happens normally in nature, at an earlier stage (Fig. 11); at higher concentrations (20 μg.%) IAA inhibited and only rhizoids were produced (Fig. 13).

Gibberellin, superimposed on the favorable concentrations of kinetin and IAA, induced a dramatic elongation. As with IAA, concentrations of gibberellin approaching the lethal induce a more rapid elongation: thus gibberellin at 100 μg.% produced the longest and thinnest filaments at 30 days growth, but growth stopped at this time and the filaments were totally bleached at 60 days (Fig. 20); only the rhizoids of the attachment disc remained green. Gibberellin at 10 μg.% elicited maximum elongation; concentrations between 10 and 40 μg.% neither inhibited nor reduced the number of green islands of cells left when the germlings bleached at the end of growth (Fig. 21). Gibberellin at 1 μg.% seemed to produce a definite response as compared with the control, but this may be due to a difference in inoculum (compare Fig. 17 with Fig. 11).

So far, the response of *Ulva* to morphogenetic substances had been to induce few or many, and shorter or longer, atypical solid filaments—a far cry from what happens in nature; still, a beginning of blade formation could be detected in the flattening at the tips of the germlings grown in kinetin 10 μg.% + IAA 5 μg.%. An elongated flat blade, probably composed of two layers of cells and similar to the one normally occurring in nature, was obtained by the addition of 20 μg.% kinetin to 3 mg.% adenine (Fig. 15). Adenine alone and lower concentrations of kinetin (2.5, 5, 10 μg.%) + adenine were completely ineffective; only rhizoids and lumpy growth around the inoculum appeared; the atypical elongated germlings were completely lacking (Fig. 14).

Discussion

Though the studies of *Ulva in* bacteria-free culture are just beginning, two unexpected findings emerge: 1) a sea weed under our experimental conditions requires exogenous hormones for normal morphogenesis; 2) the thalli, typical and atypical alike, reach only an extremely small size as compared with the natural one, then bleach, but only partially, leaving islands of green cells which, when transferred to new medium, can originate new germlings.

Thuret (1878) described only two morphological types of cells in *Ulva:* the oblong cells constituting the major portion of the thallus and the rhizoids which make up the disc of attachment. The rhizoids are formed by "tubular cells" originating in the basal part of the foliaceous thallus: these cells elongate, push their tips downward between the two cell layers of thallus, reach the substratum to which

the thallus is attached, and form a mat of filaments which anchors the thallus solidly.

Delf (1912) found that the tubular cells differ clearly from the other cells of *Ulva* in being multinucleate (they have 3–5 nuclei in the upper portion, few in the tubular portion and 2–5 nuclei in the rhizoidal portion). These observations were made on discs of *Ulva* growing on thalli of *Polysiphonia;* the material had been fixed in the early spring (*i.e.,* before the appearance of foliaceous thalli) yet the tubular cells were undoubtedly alive when fixed. Schiller (1907) believes that new germlings can originate from the rhizoids and Cotton (1910) and Delf (1912) postulate that the foliaceous part of the thallus of *Ulva* is annual while the disc of attachment is perennial.

Similarly, our experiments show that there are two types of cells: one which bleaches and dies easily, and a very resistant one. However, the resistant cells are located in two regions: 1) the disc of attachment, and 2) the erect elongated portion of the germling in which islands of cells remain green when the whole germling bleaches. Though both of these permanently green cells produce new germlings, they seem to have a different resistance to unfavorable conditions. At inhibitory concentrations of IAA (10 μg.%; Fig. 12) and gibberellin (100 μg.%; Fig. 20) no green islands appeared, all the cells of the erect part of the germling died, but the rhizoids remained green; at higher concentrations of IAA (20 μg.%; Fig. 13) the green islands of the inoculum did not produce new germlings but only a mat of rhizoids. It is most probable that the cells constituting the mat of the disc of attachment in our cultures are rhizoids, nonetheless we intend to test this hypothesis cytologically and see whether they are polynucleate. One would be tempted to consider that the cells of the green islands are also polynucleate because they are able to produce new germlings directly and without passing through the zoospore stage. However, they could also be morphologically identical with the oval cells which normally produce zoospores, but have a different potency. These cells do not appear only in the atypical germlings obtained in the laboratory; the original pieces of *Ulva,* from which our cultures derive, were small squares cut from the upper median part of the foliaceous thallus which is supposedly composed only of cells producing zoospores or gametes, yet not only zoospores were produced but a number of germlings originated directly from the piece of thallus which took the appearance, as noted, of a pincushion. Islands of permanently green cells in our cultures not only appeared in the atypical filamenteous germlings (Fig. 21), but also in the bleaching flat blade obtained with adenine + kinetin (Fig. 9). We can conclude then that another type of cell (different at least in its physiological potencies) exists among the oblong cells of the growing germlings and of the foliaceous thallus. These observations invite new studies on the morphology and potencies of the cells of *Ulva,* and on the localization and distribution in the various parts of the thallus of the various morphological and physiological types of cells. The resistant rhizoids of the attachment disc may prove the commonest and most valuable way of surviving winter and other hardships. The presence of other resistant cells in the foliaceous part of the thallus may be equally important ecologically in providing a more efficient way of spreading the species: pieces of thallus, fragmented by waves and transported by currents, can easily colonize distant sites far beyond the reach of the short-lived swimming zoospore stage.

Skoog and Miller (1957), in a penetrating review, conclude that regulation of

growth may depend more upon the quantitative interactions than upon the qualitative action of the single plant hormones. This contrasts with the previous ideas that there are specific organ-forming substances and that "determination" is an irreversible loss in the regenerative abilities of cells and tissues.

We have not yet explored separately the action of each morphogenetic substance in its active range, nor the effects of kinetin at higher concentrations nor all the various combinations of morphogenetic agents. It seems, at this stage, that production of more germlings and, especially, the elongation into atypical germlings result from the combined action of indolacetic acid and kinetin; adenine and indolacetic acid appear antagonistic.

The narrow effective range of IAA is puzzling. Judging from the elongation of the atypical filaments, kinetin and gibberellin are not toxic over a wide range, while indolacetic acid is effective only in a narrow range (1 μg.% IAA is barely active, 5 are optimal, and 10 μg.% induce rapid growth followed by rapid death). The formation of a flat thallus, so far, has been obtained by combining adenine with kinetin, but in this combination kinetin is inactive up to 20 μg.%, while in combination with indolacetic acid it elicits elongation of atypical germlings at 10 μg.% (Fig. 5). So far, only adenine + kinetin have given normal growth while growth of atypical germlings results from the combined action of kinetin and IAA.

Distinguishing between specific actions, interactions, and mixed actions of plant hormones is a complicated task in higher plants: isolated specialized tissues—an artificial situation—may be misleading for morphogenetic conclusions; mixed tissues represent different potencies, while organs are too highly specialized and reflect the interdependency of many distinct tissues.

If other algae respond to plant hormones as one may expect, they may become excellent experimental material. The Chlorophyceae, because of their closeness to the primitive land plants, may be the best choice: they abound in species representing practically all the early steps of increasing structural complexity—the simple filament; different types of heterotrichous filaments; complex branched filamentous thalli in which the prostrate and the erect system may be unequally developed; thalli with specialized oogamy; and foliaceous thalli. With algae, one can work with *whole organisms*, and not with parts of highly evolved organisms artificially avulsed from the whole, simply by selecting species in order of increasing morphological complexity. The activity of plant hormones on *Ulva* raises the question of precisely where in the algal line of evolution toward the land plants, plant hormones were first employed as morphogenetic regulators.

Earlier studies on the action of indolacetic on unicellular algae seem unconclusive or negative. Preliminary experiments, done in collaboration with J. J. Pintner and K. Gold, show that several fresh water and marine unicellular algae and even the colonial *Volvox globator* do not respond to indolacetic acid, kinetin and gibberellin: growth rate, final growth and morphology are unchanged within the concentration range effective for *Ulva*. It is not surprising that flagellates which are considered morphologically the primitive form from which the vegetal and animal tendencies of the algae have evolved, do not respond to morphogenetic hormones. Hormones are concerned with the balanced growth of a cellular organism—how can one expect to find visible changes in an organism which has no cellular parts? The lack of effect on *Volvox* supports the generally held idea that

this line is an evolutionary *cul-de-sac* and that *Volvox* is a colony of individuals. However, since the cell is the site of action of the hormones, "unicellular" algae may be the material of choice for studying the mode of action of plant hormones at the cellular level, but then, we need powerful specific antagonists to plant hormones.

It has been fortunate that plant hormones under our experimental conditions are indispensable for normal morphogenesis of *Ulva*. Quite likely this will hold for other media but, if it were not so, some nutritional factors upset the normal morphological development; the study of their role in morphogenesis could then allow a deeper insight into the action of plant hormones. ASW 8 allows better growth and the action of plant hormones is more evident in ASW 8 than in ASW III. The main difference between the two media is the presence in ASW III of an aqueous liver extract and soil extract, both of which introduce purines. Some purines, as adenine and kinetin, are important morphogenetic agents for *Ulva*, but it is conceivable that other purines may interfere with the normal processes of growth.

Definitive results can be obtained only by substituting for sea water a chemically defined medium to eliminate the unknown organic constituents of sea water.

The *Ulva* data suggest that these plant hormones may be as ecologically important for other sea weeds as vitamins are for phytoplanktonts. The auxins and gibberellins are microbial products (see Brian's review, 1957) and the unknown natural purines, which act like kinetin, may also be significantly contributed in natural waters by microbial action. It is possible therefore that the coastal zone, because of the land drainage which favors microbial growth, may never be so poor in plant hormones as to limit sea weed growth severely, but fluctuation in their level may control speed of growth and size of crop. This may be of economic importance to nations, like Japan and Ireland, which farm and use sea weeds extensively. To resolve these issues, not only are extensive pure culture studies needed but also convenient sensitive methods for assaying plant hormones in sea water.

Summary

1. Bacteria-free *Ulva lactuca*, in sea water media enriched with vitamins, grows as atypical, short, filamentous germlings which do not develop into a foliaceous thallus. These filaments reach a few millimeters, then bleach, leaving a few islands of intensely green cells which, upon transfer to new media, produce new germlings.

2. The initiation of new germlings from these green islands and the length of the atypical filaments are increased by the combination of kinetin + indolacetic acid; adenine and indolacetic acid seem antagonistic. Conspicuous elongation of the filaments is promoted by the addition of gibberellins to the kinetin-indolacetic acid combination.

3. A normal flat blade was obtained so far only with adenine + kinetin.

4. The responses depend both on the interaction and concentrations of these morphogenetic agents: indolacetic acid is effective only in a very narrow range of concentrations and a blade is produced only with relatively high concentrations of kinetin.

5. The rhizoids of *Ulva* and the cells of the green islands can produce directly new germlings and are far more resistant to unfavorable conditions than the other

cells of the thallus which can originate zoospores or gametes. The morphogenetic and ecological significance of the resistant cells is discussed.

6. These responses of a relatively simply organized sea weed to plant hormones link even more tightly the green algae to the higher land plants.

7. The variety of evolutionary steps toward increased morphological complexity in the algae suggests that whole organisms, because of their relative morphological simplicity, may be valuable experimental material for studying the mode of action of plant hormones.

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THE SO-CALLED "RECOVERY" PHENOMENON AND "PROTECTION" AGAINST X-IRRADIATION AT THE CELLULAR LEVEL

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There has been some evidence that if the sea urchin egg is allowed to remain unfertilized for several hours following x-irradiation, there will result a higher percentage of cleavage than when eggs are fertilized immediately after exposure. This has been designated as evidence of "recovery" of the egg by Henshaw (1932–1941). It presumes that such damage as is inflicted upon the egg can be rectified if the egg is given time.

Among higher forms many chemical agents have been examined to determine whether they might either "protect" the organism against x-irradiation lethality, or enhance the "recovery" from radiation insult. Among the first of the effective agents was cysteine (Patt, 1955) but more recently another —SH compound, namely cysteinamine,[2] has proven to be at least as effective. No drug has given complete protection but many have increased the dose necessary to kill a given percentage of the exposed animals, or to increase the probability of survival.

The mechanism of either the so-called "recovery" or of the chemical "protection" against x-irradiation has not been revealed. Since, among higher forms, the hematopoietic and reticuloendothelial systems are the more radiosensitive, and since the "protected" animals show quick regeneration of these systems, it has been presumed by some investigators that the so-called protective drugs somehow substituted for or masked the enzymes critically important in respiration. Since the sea urchin (Arbacia) egg showed the "recovery" phenomenon but did not have any hematopoietic system, it seemed appropriate to study the possible "protective" value of cysteinamine followed by x-irradiation and prior to fertilization of the Arbacia egg, and its possible effect on the "recovery" phenomenon.

Materials and Method

The eggs of Arbacia are readily available during the early summer and are easily fertilized to give nearly 100% cleavage and normal development. In every instance eggs from the same female were used for controls and for x-irradiation with and without benefit of the drug, so that there was no variation in the biological material. Fertilization or subsequent fixation of all the eggs of any single experiment was accomplished in 60 seconds, and in the same order in the various con-

[1] This work was done under Contract AT-30-1-GEN-70 for the Atomic Energy Commission.

[2] Cysteinamine is also known as cysteamine or beta-mercaptoethylamine or, commercially, as Becaptan hydrochloride. It was made available through Dr. W. Christiansen, representing the Labaz Laboratory, in Belgium.

tainers. The fixation was at stated intervals after insemination for the purpose of determining the cleavage percentage, and since the order of fixation was the same as the order of fertilization, and in the same elapsed time, the interval for all eggs between fertilization and fixation was the same.

It is obvious that when one deals with the cleavage percentage at a specified time after fertilization of an egg so temperature-sensitive as is the egg of Arbacia, that each set of data from the eggs of a single female on any particular day must be considered separately from other data similarly collected on other days for the simple reason of temperature variability, even though the range of temperature variation was very low. By maintaining controls for each set of data separately, effects of temperature fluctuation were obviated. This was considered to be more accurate than using a temperature control bath and averaging the data.

The eggs from a single adult Arbacia were removed by forceps and placed over cheesecloth in a beaker of filtered sea water. Within 15–20 minutes all of the mature eggs were dehisced from the ovaries and settled through the cloth into the beaker. When most of the eggs had settled on the bottom, the supernatant sea water was decanted off, and the eggs were transferred to a 500-cc. graduate which was filled with fresh, filtered sea water so that the eggs were thoroughly washed. This was repeated twice more. Finally, the eggs were diluted to 800 cc. of sea water and divided into two lots, one of which was considered the control lot. To the other was added 1 cc. of cysteamine from a freshly opened vial. In this way the concentration of eggs was the same in the control and experimental batches. The cysteamine was added within one-half hour of the beginning of irradiation so as to allow complete penetration into the egg. One cc. of cysteamine in 400 cc. of suspended eggs made a 0.0025 gram per cent solution which was non-toxic and in which eggs could be fertilized and would develop to plutei. With the above conditions, the variables were reduced to two, namely the level of exposure with or without the chemical agent.

Eggs in uniform concentration were placed in covered plastic fly boxes measuring 67 mm. in diameter and 20 mm. in depth. The controls were placed in one box and those in the chemical agent in the other, and the two boxes were superimposed during x-irradiation. When half the desired dose had been delivered, the position of the two boxes was reversed so that the slight difference in geometry of the two, with respect to the radiation, would be balanced out, and the exposure would be equated.

The x-irradiation facilities used were those provided by the Marine Biological Laboratory at Woods Hole, Mass., and consisted of two alternate-parallel x-ray tubes run at 182 KVP and 25 MA and having an equivalent filtration of 0.2 mm. of copper.[8] The output of the combined tubes at position "A" was 5,184 r/min. and at position "B" was 2,160 r/min. in air. In position "A" the distance between the targets was 18 cm. and at position "B" it was 29 cm. The maximum interval of exposure was 57.52 minutes but during this time there were brief interruptions during which eggs were removed. At all times a fan blew cool air over the dishes to dissipate any heat from the x-ray tubes. Should there have been any heat effect it would be the same for experimentals and controls. The exposures ranged from 25,000 r to 125,000 r.

[8] The author expresses here his appreciation of the efficient irradiation service rendered by Mr. Alan Brockway.

Fertilization of all eggs from a single experiment was accomplished within a period of less than one minute. At designated intervals following fertilization, samples of eggs were removed from the dishes by pipette and fixed in 10% formalin in sea water in Syracuse dishes to which had been added a small amount of acetic acid. Fixation was immediate. The order of fertilization was followed when the samples of eggs were fixed so that the time variable during the fertilization procedure was cancelled out by the same time interval and order of fixation. The percentage of cleavage was determined subsequently by counting groups of 200 eggs in each Syracuse dish, and the percentage was recorded to the nearest 5%. It was found that under the temperature conditions of the laboratory, fixation at six hours post-fertilization would include all the eggs which would ever enter cleavage, taking into consideration the matter of delay in cleavage which could be attributed to x-irradiation alone.

TABLE I

*"Recovery" in cleavage rate of Arbacia eggs following x-irradiation, due to delay in the time of fertilization**

Time: Fert. to fixation	1½ hrs.				2½ hrs.				24 hrs. Blastula
Time: Irrad. to fertilization (hrs.)	5	3	2	1	5	3	2	1	5
Controls	90	99	95	95	95	99	99	99	100**
25,000 r	75	85	60	10	85	99	90	90	100**
50,000 r	5	10	1	2	90	80	80	80	50
75,000 r	0	0	0	0	40	30	45	30	10
100,000 r	2	1	0	0	80	80	35	10	5

* Values in percentage cleaved eggs.

** See footnote No. 4.

Note: Maximum cleavage was achieved in eggs allowed to stand in fresh sea water for three hours after irradiation and before fertilization. After 100,000 r some 80% of the eggs did cleave in the time interval of two and one-third hours. The fact that at 75,000 r there was lower percentage of cleavage may indicate that some egg nuclei were functioning while at 100,000 r there may have been complete parthenogenesis.

EXPERIMENTAL DATA

The experimental data consist of percentage cleavage and development at stated intervals following delayed fertilization and also following irradiation in cysteinamine, as well as a combination of the two variables.

"Recovery": At average laboratory temperatures 50 per cent of the eggs of Arbacia will achieve the first cleavage within one hour after fertilization. If one examines fertilized eggs at one and one-third hours, the maximum percentage of cleavage will be observed. When eggs are x-irradiated and then fertilized by normal sperm, a delay in cleavage is observed, rather directly related to the level of irradiation. In the experiments reported here, eggs were x-irradiated from one

[4] The fact that there was a higher percentage of blastula than of cleavage is explained on the basis of delay in cleavage by the specific times of fixation for determining cleavage percentage. If time is not limited, the blastula percentages can be regarded as evidence of delayed but ultimate development. This apparent disparity occurred even slightly for the controls.

to five hours before insemination and the percentage cleavage was determined at one and one-third, two and one-third, three and twenty-four hours thereafter. The data are found in Table I below.

The term "recovery" has been used to describe the above phenomenon, since at any given time there is a higher percentage of cleavage in those eggs which had the greatest delay between x-irradiation and fertilization. The fact that even after 100,000 r the eggs do eventually cleave is of interest, indicating that the changes brought about by x-irradiation may be cytoplasmic, indirectly affecting the mechanisms of mitosis. In other words, the cytoplasm may show some evidence of "recovery." The mechanism for mitosis (*e.g.*, the chromosomes and spindle)

TABLE II

Cysteinamine "protection" of the Arbacia egg against x-irradiation. Effect on the time of the first cleavage

Date	Percentage cleavage (400 eggs) at 2.5 hrs. post-insemination							
	Controls		51,840 r		69,120 r		86,400 r	
	Sea wat.	Chem.	S.W.	Chem.	S.W.	Chem.	S.W.	Chem.
6/20	91	82		90	4	94	1	63
6/21	88	82	0	8	0	12	0	2
6/23	90	82	39	87	4	85	0	54
6/25	83	80	33	69	24	54	4	44
6/26			71	83	20	81	4	84
6/27			33	83	6	84	4	61
6/27			9	78	3	73	1	32
Averages:	88	82	31	70	9	69	2	48

Note: Any single experiment is complete in itself, but the data may vary with those from another day for reasons of temperature fluctuations alone. Thus, data on any horizontal line should be compared and it will be seen that in *every instance* there is a higher percentage of cleavage at 2.5 hours in eggs x-irradiated in the chemical than in the sea water alone. This is positive proof of qualified "protection" at the cellular level. The averages, below the table, have only relative significance but confirm the above.

could be contributed by the unirradiated sperm. Since none of the eggs x-irradiated above 50,000 r developed into plutei, it must be assumed that the damage to the egg nucleus is beyond "recovery," preventing normal development.

Since there is no "recovery" of the developmental potentialities it may be possible to explain the phenomenon in terms of temporary interference with insemination and fusion of the protonuclei, or with the mechanics of mitosis, the degree of interference being related to the level of x-irradiation. It is not likely that this delay is due to any effects of the x-irradiated egg, exudates, or sea water *on the spermatozoa,* for their time span of activity in dilute suspensions is very short at best. It is more probable that the egg membrane, the cytoplasm, or even the elements involved in the kinetics of pronuclear fusion and mitosis may be temporarily altered so as to delay the progression of the sperm nucleus toward the egg nucleus. Whatever this effect of x-irradiation, it is reversible in time and even

after 100,000 r some 80% of the eggs will achieve the first cleavage at least. To call this "recovery" is misleading, simply because the word generally implies much more than is demonstrated here. No egg exposed to more than 50,000 r x-rays ever "recovers" in the sense that it can develop past the critical stage of gastrulation. There is restoration of the conditions necessary for early mitosis only.

Chemical protection: Likewise the word "protection" must be qualified for in no permanent sense does the chemical "protect" animals (or eggs) against the effects of ionizing radiations, although it may lessen the effects. Nevertheless, if one x-irradiates Arbacia eggs in a tolerable solution of cysteinamine, then transfers

TABLE III

The effect of time between irradiation and fertilization as well as immersion of eggs in cysteinamine during exposure, on the percentage of cleavage (200 eggs to nearest 5%)

Time: Irradiation to fertilization	Eggs fixed 2 hours after insemination							
	6 hrs.		5 hrs.		2 hrs.		1 hr.	
	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.
Control	100	100	100	100	100	100	100	100
50,000 r	60	90	40	90	25	100	1	90
75,000 r	5	95	2	95	0	90	0	30
100,000 r	10	80	0	90	0	2	0	0
125,000 r	0	30	0	30	0	0	0	0

Note: Experimental eggs were those immersed and irradiated in the chemical while the controls were x-irradiated in filtered sea water. In *every instance* where data are available, eggs x-irradiated while in the chemical show much better percentage of cleavage than did the controls. That both "protection" by the chemical and time for "recovery" were working is indicated by the fact that at 75,000 r we have a range of cleavage 30% to 95%, depending upon the time interval between irradiation and fertilization.

Cleavage percentage values include the 2- to 8-cell stages. At 24 hours those which had been chemically "protected" during x-irradiation were normal-appearing blastulae while the control eggs were disintegrated or abnormal blastulae. Even after 125,000 r 10 per cent of the experimentals were normal-appearing blastulae while the controls were 100 per cent abnormal.

them to filtered sea water and inseminates them, there is evidence that a higher percentage of eggs will cleave than in the "unprotected" controls, no matter what level of irradiation. Table II below illustrates this point.

When one combines the two variables of time between x-irradiation and fertilization as well as the presence of the so-called "protective" agent during x-irradiation, data of Table III are obtained.

When one delays examining the eggs until about three hours after insemination, further facts become clear. An exposure of 50,000 r does not prevent cleavage in the slightest, although there is a delay. Above this level of exposure there is a drop in ultimate cleavage percentage so that at 125,000 r the maximum level of cleavage is 50% at three hours after insemination in those eggs which had an interval of five hours between irradiation and insemination. A further delay in insemination (to six hours) was deleterious so that no eggs developed.

In every instance where eggs were x-irradiated in cysteinamine, there was higher percentage of cleavage than in the "unprotected" controls. In fact, the level of cleavage was the same as that of the controls or 100% even after 125,000 r, providing there was a delay in fertilization. *Thus, we see two forces acting in favor of the egg, with additive effects up to the point of five hours delay in fertilization.*

Those eggs which were inseminated within one hour of exposure to 125,000 r, while suspended in cysteinamine, showed 90% cleavage at three hours. This is certainly evidence of better "recovery" by means of the "protective" action of the

TABLE IV

The effect of time between x-irradiation and fertilization, as well as presence of cysteinamine during exposure, on the percentage of cleavage of Arbacia eggs

Time: Irradiation to fertilization	Eggs fixed 2 hours after insemination							
	6 hrs.		5 hrs.		2 hrs.		1 hr.	
	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.
Control	100	100	100	100	100	100	100	100
50,000 r	100	100	100	100	100	100	100	100
75,000 r	80	100	80	100	90	100	80	100
100,000 r	30	100	30	100	20	90	30	90
125,000 r	0	80	50	100	40	80	10	90

Note: There is evidence from these data that cysteinamine did not alter the tendency of eggs to "recover" if time was allowed between irradiation and insemination but that the two factors were additive.

At 24 hours all controls above 50,000 r were dead while those x-irradiated to this level in cysteinamine showed 100% ciliated motile blastulae (even after 125,000 r). After 48 hours a few from 50,000 r and 75,000 r plus cysteinamine developed into crude plutei. This would never happen with "unprotected" control eggs.

drug than by time lapse between x-irradiation and fertilization. True, this was increased slightly to 100% by adding the time factor, but these figures are too close to be significantly different.

Immersion of eggs in cysteinamine following x-irradiation was invariably deleterious, the eggs going to pieces during the early attempts to cleave.

DISCUSSION

The words "recovery" and "protection" when used in radiobiology must be qualified or clearly defined.

"Recovery" has been used to imply a return to the pre-irradiated state. It is defined as a "restoration to the normal state." It is very doubtful that this ever occurs following x-irradiation-induced morphological change at the cellular level. In a complex and multi-cellular mammal, for instance, there may be "recovery" in the sense that hair returns after epilation, lymphocytes appear after lymphopenia, and even the sterile testis may again become functional. Nevertheless, in every one of these examples it is more likely that there has been regeneration from un-

damaged cells and that the originally damaged cells have been removed. The organism as a whole does have remarkable powers of restoration, but there is no evidence that the once damaged cells can themselves be "restored to the normal state."

There is no doubt (as Henshaw first pointed out) that a delay in fertilization after x-irradiation of the Arbacia egg will allow a greater percentage to undergo the early cleavages. This was confirmed up to a delay of three hours. However, ultimate cleavage was not improved and development was never achieved beyond the gastrula stage in eggs exposed to 50,000 r or more. Thus, the so-called "recovery" relates to counteracting somehow the delaying effects of x-irradiation on the mechanism of cleavage in the egg. It does not mean that the radiation-insulted egg can develop as does an unirradiated egg, a true recovery situation.

The word "protection" is also used variously (Bacq and Alexander, 1955). With mice, rats, guinea pigs, etc., it generally refers to the ability of a larger percentage of animals to survive a given dose of radiation, or to tolerate a larger dose than usual. The data are generally based upon a thirty-day survival. Certainly such animals exhibit most of the expected sequelae of x-irradiation such as shortening of life, higher incidence of cataracts, sterility, etc. It has been erroneously presumed that animals surviving thirty days after exposure are "normal" and the word "protection" is used.

Hollaender and Doudney (1955) found that when *E. coli* were x-irradiated in cysteinamine, they could tolerate twelve times as much of an exposure. That is, a dose of 60,000 r with the chemical had the same effect as 5000 r without its presence. Patt (1955) has reviewed the thesis that it is the sulfhydryl group (—SH) that is protective, by virtue of its ability to either protect or substitute for an enzyme, or to reactivate the enzyme after x-irradiation. The difficulty lies in the fact that there are many compounds containing the —SH group which have no protective value. Hollaender and Stapleton (1953) and Gray (1956) suggested that chemicals such as cysteine, entering the cell, require oxygen in order to be metabolized and thereby render the intracellular substance anoxic. But anoxia, while favoring survival of x-irradiated cells, probably does not alone explain the mechanism of cysteinamine protection.

It might be suggested here that the delay in initiation of cleavage is due to cytoplasmic effects of ionizing radiations on the egg, and those cytoplasmic effects are protected by such a chemical agent as cysteinamine. Certainly the results herein reported indicate that cleavage time is better "protected" by this chemical than by the time-lapse between x-irradiation and insemination. It is probable that the so-called "recovery" of the egg in time, with respect to initial cleavage and without benefit of chemical agent, is achieved largely by effects in the cytoplasm. One would tend to agree with Blum *et al.* (1951) that in addition to the cytoplasmic damage there is more serious damage, concerned with embryonic development, affecting the nucleoproteins. However, this is not manifest until the critical phase of gastrulation. It is of interest that the only eggs exposed to x-irradiation above 50,000 r which reached the recognizable pluteus stage were those which had been "protected" by cysteinamine and none of those benefited by a delay in insemination. This again indicates that the two mechanisms may act in a different manner but are not in conflict. In fact, they may be additive at lower levels of exposure.

Finally, it has been shown (see Bacq and Alexander, 1955) that glutathione plays a metabolic role in the process of cell division by effecting a fermentation within the cell which stimulates cell division. Specifically, cell division is not possible without the prior denaturation of protein by means of the —SH radical and the consequent reduction of the store of oxidized glutathione. Glutathione changes the oxidation-reduction level which in turn alters the fermentative metabolism that leads to cell division. The presence of thiol poisons in the cell, which could inhibit cell division, can be reversed by some of the —SH compounds, such as cysteine (Hammett, 1929) and presumably also by cysteinamine. That the —SH radical is vitally concerned in cell division has been well demonstrated.

There is no doubt that there is an increase in the soluble thiol compounds prior to cell division and also a high concentration of —SH radicals before, during and after mitosis. It is therefore conceivable that cysteinamine, like glutathione (Stern, 1956), might play a critical metabolic role in affecting mitosis. If the x-irradiated cell is given some time to repair the interference with the mitotic mechanism, or if the cell is provided with an excess of the —SH radical in the form of cysteinamine, the expected effect of cleavage delay caused by x-irradiation is not experienced. But, making available the —SH radical certainly does not allow true and full "recovery" nor is it fundamental "protection" of the cell since we would be ignoring herein all effects of x-irradiation on the chromosomes and genes which have to do with normal developmental processes. Therefore so far as development is concerned, there is neither "recovery" nor "protection."

Summary and Conclusions

1. The often used terms "recovery" and "protection" in radiobiology are specifically defined. As circumscribed, recovery at the cellular level does not occur following x-irradiation damage. Protection simply refers to better survival and in no way implies saving the exposed cell from the sequelae of x-irradiation insult.

2. The prior finding (of Henshaw) that a delay in insemination of Arbacia eggs following x-irradiation will allow for better initial cleavage percentage has been confirmed. However, it has been shown that there was no actual increase in ultimate cleavage percentage but rather, the x-irradiation factors which caused a delay in cleavage were neutralized. X-irradiated eggs never "recovered" because they could not develop through gastrulation to plutei.

3. Cysteinamine, if available to the Arbacia egg during x-irradiation, will counteract the delaying effect of x-irradiation on cleavage following insemination and will, in addition, allow further development. Some embryos will achieve the pluteus stage. This suggests that in addition to the apparent "recovery" by delay in insemination, the —SH cysteinamine must in some way reduce or prevent irradiation damage to the nucleus in some eggs, allowing them to become ciliated blastulae and even stunted plutei. They do not develop further than abnormal plutei following x-irradiations above 50,000 r, even with the benefit of the cysteinamine, so that protection of the egg to allow normal development did not occur.

4. Cysteinamine imposed on the Arbacia egg after x-irradiation was so deleterious that the early cleavage stages disintegrated rapidly even in concentrations which were tolerated well by unirradiated embryos. This may be due to the anoxia caused by cysteinamine.

5. It is concluded that "recovery" from x-irradiation damage defined as "restoration of the normal state" does not occur even with a delay in insemination. There may be some evidence of chemical nuclear "protection" to the extent that some eggs can develop to the early pluteus stage, but no further. This is hardly "protection" in the common usage of the word. It is concluded, therefore, that nuclear damage by x-irradiation is *irrevocable* and *irreparable and* that *neither "recovery" nor "protection,"* properly defined, *occurs at the cellular level following x-irradiation insult.*

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DEVELOPMENT OF PIGMENT IN THE LARVA OF THE SEA URCHIN, LYTECHINUS VARIEGATUS[1, 2]

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Very little is known concerning the origin of invertebrate pigment cells. Most invertebrate eggs contain varying amounts of carotenoid pigments, which are sufficient to mask the appearance of any new pigments. The unsegmented egg of the sea urchin *Lytechinus variegatus*, however, contains very small amounts of carotenoid or other pigment material and formation of a new pigment (echinochrome) in the early gastrula stage is readily observed. The striking appearance of pigment in early development suggests that the eggs of this animal might be unusually favorable material for a study of the cellular origin and chemo-differentiation of a defined substance—echinochrome. Echinochrome is a substituted naphthoquinone, red-purple in color, found in the test, spines, epidermis and various internal organs of sea urchins. The chemical structure and physical and chemical properties of certain echinochromes have been established by various investigators (Ball, 1936; Lederer and Glaser, 1938; Glaser and Lederer, 1939; Kuhn and Wallenfels, 1939, 1940; Wallenfels and Gauhe, 1943; Goodwin and Srisukh, 1950). A number of physiological functions have been ascribed to echinochrome. However, questions concerning the embryological and biochemical derivation, metabolism, and possible physiological functions of polyhydroxynaphthoquinones in echinoids are mainly unanswered.

The present study is an attempt to determine the embryonic origin of the echinochrome-forming cells, and to throw some light on the intracellular mechanisms affecting echinochrome synthesis.

Materials and Methods

The eggs and sperm of the sea urchin *Lytechinus variegatus* were used throughout this work. *Arbacia punctulata* was used in some instances for comparison. The animals were collected in the Gulf of Mexico at the mouth of Alligator Harbor, Florida, using the facilities of the Florida State University Marine Laboratory. The animals were maintained in running sea water or jugs of continually aerated sea water at about 15 degrees centigrade.

The eggs and sperm were obtained from the animals by the KCl injection method. Eggs were fertilized in filtered sea water with approximately 0.5% sperm suspensions.

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Development was observed with the dissecting microscope and the phase contrast microscope. The ultra violet absorption spectrum of the echinochrome was determined by means of the Beckman DU Spectrophotometer.

All results are based on experiments involving at least 200 eggs. Each experiment was repeated at least twice. Any deviation from these numbers will be discussed in the text. The various techniques used in the individual experiments will be described with the results of the experiments for the sake of continuity and clarity.

Results

Normal development

The fertilized egg of *Lytechinus* is relatively pigment-free. There is a very faint yellowish cast to the egg, probably due to carotenoids as in most echinoderm eggs, but the nucleus and cellular inclusions are clearly visible under the dissecting microscope.

The echinochrome-containing cells (echinophores) are first noticeable under the microscope during early gastrulation, when the veg_2 cell layer is invaginating to form the gut. They appear to be in the ectodermal layer and at first only in the region of the invagination. As gastrulation proceeds, the echinophores become dispersed throughout the gastrula, apparently in the ectodermal layer. In the pluteus, these cells are evenly dispersed in the outer body wall, with usually some concentration in the arm tips.

A series of experiments was designed to determine more precisely the origin of these cells in the course of development of the embryo and perhaps what intra- or inter-cellular mechanisms were involved.

Lithium experiments

Exogastrulae were produced using the technique of Herbst (1892) (treatment of fertilized eggs in a 0.1 *M* LiCl solution in sea water for five hours). This presented an opportunity to determine the effect of LiCl on pigmentation and to determine if the normal association of the germ layers is necessary for pigmentation. These exogastrulae were striking in that echinophores always appeared in the ectodermal portion of the larvae and never in the endoderm. The pigment appeared first at the site of evagination and by the time the evaginated gut was completely formed these cells were dispersed throughout the ectodermal wall of the larvae. The evaginated gut wall never contained pigment cells, although occasionally pigment could be seen inside the lumen where apparently it had migrated. This particular phenomenon will be discussed in more detail later, in connection with the amoeboid movements of these cells. From this experiment it may be said that pigment is associated with the ectoderm (at least in exogastrulae) and that a normal association of the germ layers is not necessary for pigment formation. LiCl in the concentrations (0.1 *M*–0.5 *M*) used had no apparent effect on pigmentation.

Thiocyanate experiments

Since the pigment cells were found only in the ectodermal portions of the LiCl-induced exogastrulae, it would be interesting to know if the ectoderm alone is able to give rise to these echinophores.

"Dauer" blastulae (permanent blastulae with no gut or skeleton) were formed using NaSCN (0.125 *M*) and the technique of Lindahl (1936). Those embryos which developed into "Dauer" blastulae were never pigmented. They remained as colorless hollow balls of ciliated ectodermal cells, whereas if there was any sign of invagination and appearance of endodermal derivatives, pigment was always formed in the ectodermal regions. In these cases, where very little endoderm was present, pigment cells were few and widely scattered. However, as in previous experiments, the pigment first appeared around the invaginating region, and later gradually became dispersed throughout the larval wall.

Apparently the ectoderm alone is not capable of giving rise to pigment, at least not in those "Dauer" blastulae formed by treatment with NaSCN.

Isolation experiments

It is possible to study the effects of ectodermization on pigmentation, without the influence of a chemical agent such as NaSCN. In these experiments, the technique of Hörstadius (1928) was used. The most satisfactory method for removing the fertilization membranes was found to be the shaking method of Driesch (1891).

Since *Lytechinus* eggs have no pigment band and removal of the fertilization membrane also removes the polar bodies at the animal pole, there is no sure way of determining which is the animal and which is the vegetal pole at the 8-cell stage. However, at the 16-cell stage, the micromeres have appeared and identification of the vegetal pole is quite easy. For this reason, most of the operations were done at the 16-cell stage. Some, however, were done at the 8-cell stage and will be discussed later. About 10 eggs at the 16-cell stage were placed in a drop of sea water in the shallow depression in the lid of a small stender dish, by means of a capillary pipette. The drop of sea water was kept as small as possible so that the surface tension served to hold the embryos in place while the cuts were being made. Under the dissecting microscope the desired blastomeres were separated with glass dissecting needles. The separated cells were picked up with the capillary pipette, transferred to sea water in syracuse dishes and allowed to develop.

By means of this technique, 30 animal and 30 vegetal halves were isolated at the 16-cell stage. Invariably, the animal halves formed typical "Dauer" blastulae, while the vegetal halves gastrulated and sometimes formed miniature plutei. These were usually defective in that they often had only one arm or a poorly formed gut and skeleton. The "Dauer" blastulae from isolated animal halves were never pigmented and remained unpigmented until they died. The vegetal half-embryos were always pigmented in a typical fashion.

Similar results were obtained in 40 cases in which halves of the 8-cell stage were isolated. However, since there was no way of identifying the animal and vegetal halves, the assumption was made that some of the cuts would isolate halves containing two animal and two vegetal cells, depending on the plane of the cut. This assumption appeared to be well founded, since some halves formed "Dauer"

blastulae, while most developed into pigmented plutei. Only the animal halves form "Dauer" blastulae whereas the vegetal halves and those halves containing two animal and two vegetal cells gastrulate and develop pigment.

These experiments confirmed the results obtained in the NaSCN experiments, that is, that the ectoderm alone does not produce pigment. Pigment is formed when the embryo gastrulates (regardless of what fraction of the original egg is present) and only if the embryo gastrulates.

Vital staining experiments

In order to determine exactly what cells are responsible for the production of these echinophores, the technique of vital staining was employed. The technique of Hörstadius (1935) was used. The dye used was Grüblers "Neutral Rot," since this was the only dye found to penetrate the larvae satisfactorily.

The eggs were stained at the 16-cell stage. A fine glass capillary which was filled with agar containing a one per cent solution of neutral red was put into a drop of sea water in a stender dish lid. The egg to be stained was moved into position and held in place for about one minute, at which time enough dye was absorbed to render it readily identifiable. At the 16-cell stage the eight animal cells were stained in this way in a series of 12 eggs. These stained cells never became pigmented after gastrulation. The four micromeres in 12 eggs were stained in the same way and here, too, the stained cells never produced echinophores. When the four macromeres (of 12 eggs) were stained, the pigment cells in the pluteus showed traces of the dye. The macromeres do not divide until the end of the 32-cell stage, so that staining of the veg_1 and veg_2 cells derived from the macromeres is impossible until the 64-cell stage. At the time of the 64-cell stage, the individual blastomeres are extremely small and difficult to stain individually. However, an attempt was made to stain the cells comprising veg_1 in one series of eight eggs, and veg_2 in another series of eight eggs. It was found that the veg_2 cells and the micromere material always invaginated at gastrulation and gave rise to no pigmented cells. Although it was almost impossible to stain only veg_1 cells since some of the stain invariably got into veg_2 cells, it was none the less possible to see that it was from this material that the echinophores ultimately arose, since the concentration of the dye in the veg_1 cells was much greater. The material from veg_1 did not invaginate, but at the time of gastrulation, it remained near the site of invagination and ultimately gave rise to the ventral ectoderm of the pluteus.

Therefore, it would seem that the echinophores originate from the veg_1 cells but only under conditions permitting axial differentiation. The question then arises as to how these pigment cells become dispersed throughout the ectoderm of the pluteus, if their origin is localized in material which is ultimately destined to become only the ventral ectoderm of the pluteus. A series of observations gave an answer to this question.

Phase microscope observations

Boveri (1901) noticed amoeboid cells, of a brick red color, appearing in the late gastrula stage of the sea urchin *Paracentrotus lividus*. He considered the

pigment the same as that in the egg. Monroy *et al.* (1951) also noted these amoeboid cells and suggested the possibility that the pigment in them might be echinochrome, although they were not able to obtain enough material to prove its presence. Neither of these workers reported a detailed study of the movements of the pigment cells, or their location.

These cells were studied in *Lytechinus variegatus* under the phase contrast microscope. In the early gastrulae when the echinophores first appeared, it was found that the cells were large, irregularly shaped structures in the region of the invagination. This would be the material derived from veg_1 cells. First, small pale orange pigment granules appeared. These were not easily visible except under high magnification. After a few hours, when the invagination process was almost complete, the pigment in these cells became darker red in color and much more concentrated, while the cells themselves increased greatly in number. At this time, it could be seen that the pigment cells were amoeboid in nature, apparently able to move freely within the ectodermal layer in any dimension. However, they were never seen to enter the endodermal layer underneath. By the pluteus stage, they had invaded the ectoderm of the larva and appeared to be most concentrated in the more actively growing arm tips of the pluteus. By the time the pluteus was fully formed, the echinophores had become much less motile and migration had virtually ceased.

In observations of eight exogastrulae, the same pattern was seen, except that the endoderm was no longer immediately under the ectoderm. The echinophores were seen occasionally to work themselves completely free of the ectoderm and come to lie in the cavity of the blastocoel and eventually even in the everted gut lumen. This explains the occasional appearance of pigment in the gut cavity of exogastrulae.

The next series of experiments was designed to determine what physical and chemical factors in the developing egg were responsible for the formation of the pigment and to obtain some information as to the relative roles played by the nucleus and cytoplasm in this synthesis. Since the pigment is elaborated long before the organism begins to take in food material from the outside, the pigment must be synthesized from pre-existing materials present in either the egg or sperm, or both.

Hybridization experiments

A series of experiments was designed to determine the effect of *Arbacia* × *Lytechinus* hybridization on subsequent pigmentation. Tennents' (1912) method (ageing of gametes for two hours followed by a five-minute treatment with alkaline sea water before fertilization) was used in obtaining these hybrids. The larvae from *Arbacia* male × *Lytechinus* female were all maternal in appearance. Pigmentation, size and general body structure of gastrulae (very few reached the pluteus stage) were typical of *Lytechinus*. The reverse cross, *Lytechinus* sperm × *Arbacia* egg, was unsuccessful in that no gastrulae were obtained and nothing could be seen concerning any newly formed pigment cells at gastrulation. These experiments were repeated three times with practically identical results.

It can be said only that *Arbacia* sperm cannot affect normal pigmentation in the

Lytechinus egg, in *Arbacia* sperm × *Lytechinus* egg hybrids. The question then arises as to the normal role, if any, played by the sperm in pigmentation.

One way of answering this question is to study the development of artificially activated eggs.

Parthenogenesis experiments

The (butyric acid-hypertonic sea water) method of Tennent (1912) was found to be the most satisfactory for producing parthenogenetic larvae. Again, it was noted that if gastrulation occurred, pigment was formed and apparently in the same fashion as described for normal fertilized eggs. The parthenogenetic plutei were normally pigmented and quite like the fertilized controls. These experiments were also repeated three times with the same results. It would seem that the presence of the sperm cell is not essential for pigment formation.

Identification of the pigment

The assumption has been made so far that the pigment in question is the substituted naphthoquinone, echinochrome. The proof of this lies in isolation, physical and chemical properties and spectrophotometric analysis. Echinochrome may be extracted by treatment with slightly acidified, organic solvents such as 80 per cent acetone or ether containing one per cent of HCl. The pigment may be then transferred by dilution into diethyl ether and chromatogrammed to remove impurities. The free compound shows very slight solubility in water or petroleum ether but is readily restored by shaking in air or by any of a number of mild oxidizing agents (Ball, 1936).

Clearly defined absorption bands are exhibited by solutions of echinochrome in various solvents. According to Kuhn and Wallenfels (1939), the absorption maxima of an echinochrome solution in carbon disulphide were 535, 499 and 464 mμ, in chloroform 532, 497 and 462 mμ, in benzene 532, 494 and 461 mμ and in concentrated sulfuric acid, 502 and 469 mμ.

The following evidence shows that the pigment appearing at gastrulation in the sea urchin, *Lytechinus variegatus,* has the properties of echinochrome.

(1) The pigment is orange-red in color.
(2) The pigment may be extracted from gastrulae and plutei by the above described procedure.
(3) The pigment, when extracted and dried, is nearly insoluble in water and petroleum ether.
(4) The pigment turns red in acid solution and violet in the alkaline range.
(5) The pigment is soluble in diethyl ether, acetone, ethanol and carbon disulfide.
(6) The pigment is very slightly soluble in chloroform.
(7) The addition of 5 mg. of sodium hydrosulfite to 10 ml. of a brick red solution of the pigment quickly bleaches the solution.
(8) The addition of small amounts of an oxidizing compound (hydrogen peroxide) or shaking in air quickly restores the color to the solution.
(9) The absorption spectrum of a carbon disulfide-pigment solution showed peaks on the Beckman DU Spectrophotometer at 530, 491, and 460 mμ.

Carotenoids, chromolipids, melanins and flavins may also be reddish in color (Sumner and Doudoroff, 1943). However, melanins and flavins (Mayer and Cook, 1943) are insoluble in almost all organic solvents. Flavins are water-soluble. The carotenoids may be bleached (Fox, 1936) but only by oxidizing agents rather than reducing agents. The absorption spectrum is typical of echinochrome and not carotenoids, since the carotenoid absorption peaks are around 510 and 485 mμ (Fox and Scheer, 1941). The change in color with changes in pH is also typical of quinone pigments. It was not possible to isolate and crystallize a sufficient amount of the pigment from plutei to permit further analysis of its physical and chemical properties but on the basis of the above evidence, the pigment appears to be echinochrome.

Chemo-differentiation study

The effect of a large number of inhibitors was studied in an attempt to specifically inhibit pigmentation and perhaps learn somethong of the metabolic pathway involved in its synthesis. It was found, however, that only those inhibitors which stopped development at gastrulation or ectodermized the eggs, such as 2-4-dinitrophenol, pyocyanine and iodosobenzoic acid, had an effect on pigmentation.

Discussion

Boveri (1901) first noticed in the late gastrula stage of the sea urchin *Paracentrotus lividus* the appearance of amoeboid cells, rather heavily loaded with large pigment granules which differed from those of the unsegmented egg both with respect to their larger size and to their color. He noted that the number of these cells increased rapidly with the age of the embryo. Boveri, however, considered the pigment of the same nature as that of the egg. Monroy *et al.* (1951) noted that starting from the stage when the new pigment appeared, the embryos still retained a red-violet color after having been exhaustively extracted for carotenoids and that the remaining color was due to pigment still present in the amoeboid cells. These workers showed that the pigment could not be extracted with chloroform, acetone, methanol or pyridine but on slight acidification with dilute HCl, it could be taken up quantitatively in ether. It was thought the pigment was probably echinochrome but they were not able to obtain sufficient amounts to prove it spectrophotometrically. They also noted that the eggs of one female developed normally up to the beginning of gastrulation when exogastrulation occurred. In this case, they were unable to detect echinochrome. This observation seems unlikely in view of the exogastrulation experiments of the present study and was probably due to masking by other pigment.

Gustafson and Lenique (1951), using *Psammechinus miliaris,* mentioned pigment formation in the gastrula stage. They did not identify the pigment. However, they did mention that the red pigment cells became especially concentrated in the arm tips and the apical region, where the ectoderm is characterized by high mitochondrial activity. This observation is confirmed in the present study as previously mentioned, where echinophores are concentrated in the arm tips and apical region. It was also noted that in advanced starving plutei, the amount of echinochrome is appreciably reduced, suggesting the use of this protein-echinochrome complex as a food source under extreme conditions.

Using the *Lytechinus* egg in which the pigment is not sufficient to mask pigment elaboration subsequent to fertilization, it is possible to trace the differentiation of chromatophores with considerable accuracy. It was found that a pigment was synthesized in the embryo in the gastrula stage. This pigment was echinochrome. The particular cells in which the pigment appeared were shown by vital staining to originate from veg_1 and to be amoeboid in nature. With the use of isolation techniques and chemical treatment it was shown that pigment formation occurred only in association with gastrulation. Evidently, pigment cell differentiation is related to gastrulation in some way. Since the pigment cells differentiate in exogastrulae as well as in normal embryos, the differentiation does not appear to be an induction effect, at least to the extent that it requires the juxtaposition of endoderm with the other germ layers during gastrulation. It appears more likely that pigment cell differentiation, including the formation of pigment itself, is under the same control system as that governing the differentiation of other parts of the embryo (*e.g.*, skeleton, muscle, gut, coelom, etc.). From the work of Runnström (1933) and especially Hörstadius (1939) this over-all differentiation appears to depend upon the quantitative interaction of some sort of double gradient system. This system may function to produce pigment cells from veg_1 in normal development, but when the system is modified experimentally, for example by surgical or chemical treatments that result in "Dauer" blastulae, then pigment cells as well as other types of tissue fail to differentiate.

The production of echinochrome by the differentiating chromatophores presents an interesting problem in the chemo-differentiation of a defined substance. Unfortunately, no information is available concerning the pathways of echinochrome synthesis. However, echinochrome production may be correlated with protein synthesis, at least to the extent that new enzymes required for echinochrome synthesis may be elaborated by the embryo. Furthermore it is known that echinochrome occurs in the form of a protein complex (Kuhn and Wallenfels, 1940; Shapiro, 1946), and extensive protein synthesis begins at the same time that echinochrome first appears in the embryo, namely, at the time of gastrulation (Caspersson, 1947; Brachet, 1941; Zeuthen, 1951; Hultin, 1950; Perlmann, 1954). It is of interest to note the similarities between melanophore development in the vertebrates and echinophore development in the sea urchin. DuShane (1935) proved the neural crest origin of pigment cells in the amphibian. The formation of the neural crest and subsequent pigmentation are dependent on gastrulation in the amphibian and both amphibian and *Lytechinus* pigment cells are ectodermal in origin. In the amphibian, however, pigment cell formation is more complex, in that gastrulation induces the formation and differentiation of the neural crest, which in turn differentiates still further, giving rise to a number of structures, among which are the pigment cells. These cells, too, are amoeboid and migrate to their definitive position (Twitty and Niu, 1954) where they apparently lose their amoeboid capabilities and come to rest.

Summary

1. A pigment having the properties of echinochrome is synthesized in the embryo of the sea urchin *Lytechinus variegatus*. Differentiation of the echinophores and synthesis of the echinochrome begins at the gastrula stage.

2. Echinophores differentiate from the veg_1 cell layer of the embryo, become amoeboid and migrate into other ectodermal regions.

3. Echinophore differentiation appears to depend upon a normal relation of the "double gradient" system of the embryo. Since echinophores were produced in exogastrulae, normal juxtaposition of the germ layers is not essential.

4. The sperm nucleus was found to have no essential role in the pigmentation process. Pigment formed according to the maternal pattern in hybrid and parthenogenetic embryos.

5. Of a variety of chemical substances tested, including several respiratory and other inhibitors, only those agents which inhibited gastrulation of the embryo caused failure of pigment formation.

6. Echinochrome synthesis is apparently related to protein synthesis in the embryo.

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